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(54) Title: METHOD FOR THE PURIFICATION OF ALBUMIN CONJUGATES

(57) Abstract: A method for separating albumin conjugates from unconjugated albumin in a solution comprising albumin conjugates and unconjugated albumin by hydrophobic Interaction Chromatography (HIC). The solution is loaded onto the hydrophobic column equilibrated in aqueous buffer having a high salt content; applying to the column a gradient of decreasing salt concentration; and collecting the eluted albumin conjugates.

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METHOD FOR THE PURIFICATION OF ALBUMIN CONJUGATES

BACKGROUND OF THE INVENTION

(a) Field of the Invention

[0001] This invention relates to a method of purification for isolating albumin conjugates from a solution comprising both albumin conjugates and unconjugated albumin.

(b) Description of Prior Art

[0002] WO 95/10302 and WO 99/24074 describe the formation of conjugates of albumin wherein the molecule of interest has a reactive functionality coupled thereto that is adapted to covalently bond to albumin, thus forming a conjugate. These conjugates can be formed *in vivo*, but they can be formed *in vitro* as well. The formation of the conjugate *in vitro* involves the addition of a molecule coupled to a reactive functionality to a solution of albumin. The primary end products from this reaction are unconjugated albumin, the albumin conjugate and the unreacted molecule coupled to the reactive functionality.

[0003] It would be highly desirable to be provided with a method for purifying albumin conjugate from a solution comprising albumin conjugate and unconjugated albumin.

SUMMARY OF THE INVENTION

[0004] In accordance with the present invention there is provided a method for separating albumin conjugate from unconjugated albumin in a solution comprising albumin conjugate and unconjugated albumin, the method comprising:

a) loading the solution onto a hydrophobic solid support equilibrated in aqueous buffer having a high salt content;

b) applying to the support a gradient of decreasing salt content; and

c) collecting eluted albumin conjugate.

[0005] In a preferred embodiment of the present invention, the albumin conjugate consists of a molecule having a Michael acceptor covalently coupled thereto which bonds to albumin, and more preferably the bond is between the Michael acceptor and cysteine 34 of albumin.

[0006] In a more preferred embodiment of the present invention, the Michael acceptor is a maleimide group, and more preferably, the maleimide group is maleimid-propionic acid (MPA). The Michael acceptor is optionally coupled to the molecule via a linker. The linker is preferably selected in the group consisting of hydroxyethyl motifs such as (2-amino) ethoxy acetic acid (AEA), ethylenediamine (EDA), 2-[2-(2-amino)ethoxy)] ethoxy acetic acid (AEEA), amino ethoxy ethyl amino succinic acid (AEEAS); one or more alkyl chains (C1-C10) motifs such as glycine, 3-aminopropionic acid (APA), 8-aminooctanoic acid (AOA), octanoic acid (OA), 4-aminobenzoic acid (APhA). Preferred linkers are OA, ADE, AEA, AEEA and AEEAS. A combination of two linkers can also be used such as, for examples, AEEA-EDA, AEEA-AEEA, AEEAS-AEEAS, and AEA-AEEA.

[0007] In a preferred embodiment of the present invention, the albumin is selected from the group consisting of serum albumin, recombinant albumin and albumin from a genomic source.

[0008] In a preferred embodiment of the present invention, the albumin is selected from the group consisting of human albumin, rat albumin, mouse albumin, swine albumin, bovine albumin, dog albumin and rabbit albumin, more preferable human serum albumin.

[0009] In a preferred embodiment, albumin is modified with at least one selected from the group consisting of fatty acids, metal ions, small

molecules having high affinity to albumin, and sugars, such as, but not limited to, glucose, lactose and mannose.

[0010] In a preferred embodiment of the present invention, the molecule is selected from the group consisting of a peptide, DNA, RNA, small organic molecule and a combination thereof. The peptide has preferentially a molecular weight of at least 57 daltons. The peptide is intended to include, but not being limited to, GLP-1, GLP-2, ANP, K5, dynorphin, GRF, insulin, natriuretic peptides, T-20, T-1249, C-34 and PYY. The small molecule is intended to include, but not being limited to, vinorelbine, gemcitabine and paclitaxel. In a more preferred embodiment of the present invention, when the molecule is a DNA, RNA or a small organic molecule, it is covalently attached to the albumin through an acid sensitive covalent bond or a peptide sequence susceptible to proteolytic cleavage, thereby allowing the separation of the molecule from albumin and the entry of the molecule into a cell.

[0011] In a preferred embodiment of the present invention, the hydrophobic solid support is a column containing a hydrophobic resin such as, but not limited to, octyl sepharose, phenyl sepharose and butyl sepharose and more preferably butyl sepharose.

[0012] In another embodiment of the present invention, the hydrophobic solid support comprising a hydrophobic ligand such as Cibacron Blue F3G-A, ether or isopropyl groups in association with a support such as polystyrene/divinyl benzene matrix.

[0013] Substances are separated on the basis of their varying strengths of hydrophobic interactions with hydrophobic ligands immobilized to an uncharged matrix. This technique is usually performed with moderately high concentrations of salts (1M) in the start buffer (salt promoted adsorption). Elution is achieved by a linear or stepwise decrease in salt concentration.

[0014] The type of ligand, the degree of substitution, the pH and the type and concentration of salt used during the adsorption stage have a profound effect on the overall performance (e.g. selectivity and capacity) of a HIC matrix (Hydrophobic Interaction Chromatography matrix).

[0015] The solvent is one of the most important parameters which influence capacity and selectivity in HIC (Hydrophobic Interaction Chromatography). In general, the adsorption process is more selective than the desorption process. It is therefore important to optimize the start buffer with respect to pH, type of solvent, type of salt and concentration of salt. The addition of various "salting-out" salts to the sample promotes ligand-protein interactions in HIC. As the concentration of salt is increased, the amount of bound protein increases up to the precipitation point for the protein. Each type of salt differs in its ability to promote hydrophobic interactions. The influence of different salts on hydrophobic interaction follows the well-known Hofmeisters series found below:

Hofmeisters series

Salting-out effect

Anions :

$PO_4^{3-} > SO_4^{2-} > CH_3COO^- > Cl^- > Br^- > NO_3^- > ClO_4^- > I^- > SCN^-$

Chaotropic effect

Cations :

$NH_4^+ < Rb^+ < K^+ < Na^+ < Cs^+ < Li^+ < Mg^{2+} < Ba^{2+}$

[0016] Increasing the salting-out effect strengthens the hydrophobic interactions, whereas increasing the chaotropic effect weakens them. Therefore, ammonium sulfate exhibits a stronger salting-out effect than sodium chloride. The most commonly used salts for HIC are ammonium sulfate ($(NH_4)_2SO_4$), sodium sulfate ($(Na)_2SO_4$)), magnesium sulfate

($MgSO_4$), sodium chloride (NaCl), potassium chloride (KCl), and ammonium acetate (CH_3COONH_4).

[0017] Protein binding to HIC adsorbents is promoted by moderate to high concentrations of "salting-out" salts, most of which also have a stabilizing influence on protein structure due to their preferential exclusion from native globular proteins, i.e. the interaction between the salt and the protein surface is thermodynamically unfavorable. The salt concentration should be high enough (e.g. 500-1000 mM) to promote ligand-protein interactions yet below that which causes precipitation of the protein in the sample. In the case of albumin, the salt concentration should be kept below 3M (moles per liter). The principle mechanism of salting-out consists of the salt-induced increase of the surface tension of water (Melander and Horváth, 1977). Thus, a compact structure becomes energetically more favorable because it corresponds to smaller protein-solution interfacial area.

[0018] Interestingly, we found that under the same conditions (i.e. buffer composed of SO_4^{2-}, PO_4^{2-} or CH_3COO^- with any counter ion), these salts exhibit their salting-out effect upon essentially all conjugated albumin described herein in a manner different to non-conjugated albumin (i.e. mercaptalbumin and albumin capped with cysteine), thus enabling a consistent chromatographic separation between conjugated albumin versus non-conjugated albumin. That is, we observe that lower concentrations of salt are required to promote interactions between ligand and conjugated albumin than between ligand and non-conjugated albumin. This chromatographic separation is essentially independent of (a) the sequence of albumin (e.g. human, mouse, rat, etc.) (b) the source of albumin (i.e. plasma derived or recombinant) (c) the molecular weight of the conjugated molecule, (d) the position of the Michael acceptor (or maleimide group) within the structure of the molecule, (e) the peptide sequence or chemical structure of the molecule, and (f) the three-

dimensional structure of the conjugated molecule, e.g. linear versus loop structure.

[0019] In a preferred embodiment of the present invention, the salt of the aqueous buffer has a sufficient salting out effect. For providing a sufficient salting out effect, the salt is preferably, but not limited to, phosphate, sulfate and acetate. More preferably, the salt is phosphate or sulfate. The selection of the cation of the buffer is less critical and therefore, such cation can be selected, without limitation, from the group consisting of NH_4^+, Rb^+, K^+, Na^+, Cs^+, Li^+, Mg^{2+} and Ba^{2+}.

[0020] The aqueous buffer is preferably ammonium phosphate, ammonium sulfate and magnesium phosphate, and more preferably ammonium sulfate.

[0021] In a preferred embodiment of the present invention, the buffer pH is between 3.0 and 9.0; more preferably between 6.0 and 8.0, and even more preferably, the pH is 7.0.

[0022] In a preferred embodiment of the present invention, the buffer and the hydrophobic solid support are at room temperature (about 25°C) or at 4°C or in between.

[0023] Table 1 shows an example of the effect of varying salts for purification of preformed HSA:first GLP-1 analogue conjugate from a solution of HSA using butyl-sepharose resin (structure of the first GLP-1 analogue is described in Example 1 below).

Table 1

Salt type	Starting salt concentration of 750mM	Starting salt concentration of 1,750 mM
Ammonium phosphate	Yes	yes
Ammonium sulfate	Yes	yes
Ammonium chloride	No	no
Ammonium iodide	No	no
Ammonium thiocyanate	No	no

Magnesium sulfate	No	yes
Magnesium phosphate*	-	-
Barium sulfate*	-	-

* means that the salt is not soluble at concentrations of 1750 mM or 750 mM in 20mM sodium phosphate (pH 7), 5mM caprylate

Yes means that successful resolution is achieved between the HSA:first GLP-1 analogue conjugate and the non-conjugated HSA

No means that no separation is achieved between the HSA:first GLP-1 analogue conjugate and the non-conjugated HSA

[0024] The term "peptide" is intended to mean an amino acid sequence having a molecular weight of at least 57 daltons. The peptidic sequence can be circular (loop structure) such as ANP, may contain more than one amino acid chain such as insulin or may be linear such as K5, dynorphin A, C-34 and GLP-1.

[0025] All references herein are hereby incorporated by reference.

BRIEF DESCRIPTION OF THE DRAWINGS

[0026] Fig. 1 illustrates the purification of the conjugate HSA:first GLP-1 analogue (SEQ ID NO:1) by a preferred embodiment of the method of the present invention;

[0027] Fig. 2 illustrates the purification of the conjugate HSA:first GRF analogue (SEQ ID NO:2) by a preferred embodiment of the method of the present invention;

[0028] Fig. 3 illustrates the purification of non-conjugated HSA by a preferred embodiment of the method of the present invention;

[0029] Fig. 4 illustrates the purification of the conjugate rHSA:first GLP-1 analogue (SEQ ID NO:1) by a preferred embodiment of the method of the present invention;

[0030] Fig. 5 illustrates the purification of HSA cortex by a preferred embodiment of the method of the present invention;

[0031] Fig. 6 illustrates the purification of the conjugate HSA:K5 analogue (SEQ ID NO:3) by a preferred embodiment of the method of the present invention;

[0032] Fig. 7 illustrates the purification of the conjugate HSA:first insulin derivative (SEQ ID NO:4) by a preferred embodiment of the method of the present invention;

[0033] Fig. 8 illustrates the purification of the conjugate HSA:second insulin derivative (SEQ ID NO:5) by a preferred embodiment of the method of the present invention;

[0034] Fig 9 illustrates the purification of the conjugate HSA:first C34 analogue (SEQ ID NO:6) by a preferred embodiment of the method of the present invention;

[0035] Fig 10 illustrates the purification of the conjugate HSA:second C34 analogue (SEQ ID NO:7) by a preferred embodiment of the method of the present invention;

[0036] Fig. 11 illustrates the purification of the conjugate HSA:third C34 analogue (SEQ ID NO:8) by a preferred embodiment of the method of the present invention;

[0037] Fig. 12 illustrates the purification of L-cysteine by a preferred embodiment of the method of the present invention;

[0038] Fig. 13 illustrates the purification of L-cysteine:first GLP-1 analogue (SEQ ID NO:1) by a preferred embodiment of the method of the present invention;

[0039] Fig. 14 illustrates the purification of the conjugate HSA:second GLP-1 analogue (SEQ ID NO:9) by a preferred embodiment of the method of the present invention;

[0040] Fig. 15 illustrates the purification of the conjugate HSA:third GLP-1 analogue (SEQ ID NO:10) by a preferred embodiment of the method of the present invention;

[0041] Fig. 16 illustrates the purification of the conjugate HSA:fourth GLP-1 analogue (SEQ ID NO:11) by a preferred embodiment of the method of the present invention;

[0042] Fig. 17 illustrates the purification of the conjugate HSA:fifth GLP-1 analogue (SEQ ID NO:12) by a preferred embodiment of the method of the present invention;

[0043] Fig. 18 illustrates the purification of the conjugate HSA:first Exendin-4 analogue (SEQ ID NO:13) by a preferred embodiment of the method of the present invention;

[0044] Fig. 19 illustrates the purification of the conjugate HSA:second Exendin-4 analogue (SEQ ID NO:14) by a preferred embodiment of the method of the present invention;

[0045] Fig. 20 illustrates the purification of HSA:MPA by a preferred embodiment of the method of the present invention;

[0046] Fig. 21 illustrates the purification of HSA by a preferred embodiment of the method of the present invention;

[0047] Fig. 22 illustrates the purification of the conjugate HSA:second C34 analogue (SEQ ID NO:3) by a preferred embodiment of the method of the present invention;

[0048] Fig. 23 illustrates the purification of the conjugate HSA:first Dynorphin A analogue (SEQ ID NO:15) by a preferred embodiment of the method of the present invention;

[0049] Fig. 24 illustrates the purification of the conjugate HSA:first ANP analogue (SEQ ID NO:16) by a preferred embodiment of the method of the present invention;

[0050] Fig. 25 illustrates the purification of the conjugate HSA:second Dynorphin A analogue (SEQ ID NO:17) by a preferred embodiment of the method of the present invention;

[0051] Fig. 26 illustrates the purification of the conjugate HSA:ACE inhibitor (SEQ ID NO:18) by a preferred embodiment of the method of the present invention;

[0052] Fig. 27 illustrates the purification of the conjugate HSA:sixth GLP-1 analogue (SEQ ID NO:19) by a preferred embodiment of the method of the present invention;

[0053] Fig. 28 illustrates the purification of the conjugate HSA:seventh GLP-1 analogue (SEQ ID NO:20) by a preferred embodiment of the method of the present invention;

[0054] Fig. 29 illustrates the purification of the conjugate HSA:eighth GLP-1 analogue (SEQ ID NO:21) by a preferred embodiment of the method of the present invention;

[0055] Fig. 30 illustrates the purification of the conjugate HSA:ninth GLP-1 analogue (SEQ ID NO:22) by a preferred embodiment of the method of the present invention;

[0056] Fig. 31 illustrates the purification of the conjugate HSA:tenth GLP-1 analogue (SEQ ID NO:23) by a preferred embodiment of the method of the present invention ;

[0057] Fig. 32 illustrates the purification of the conjugate HSA:eleventh GLP-1 analogue (SEQ ID NO:24) by a preferred embodiment of the method of the present invention;

[0058] Fig. 33 illustrates the purification of the conjugate HSA:third Exendin-4 analogue (SEQ ID NO:25) by a preferred embodiment of the method of the present invention;

[0059] Fig. 34 illustrates the purification of the conjugate HSA:twelfth GLP-1 analogue (SEQ ID NO:26) by a preferred embodiment of the method of the present invention;

[0060] Fig. 35 illustrates the purification of the conjugate HSA:first insulin derivative (SEQ ID NO:4) by a preferred embodiment of the method of the present invention;

[0061] Fig. 36 illustrates the purification of the conjugate HSA:third insulin derivative (SEQ ID NO:27) by a preferred embodiment of the method of the present invention;

[0062] Fig. 37 illustrates the purification of the conjugate HSA:second insulin derivative (SEQ ID NO:5) by a preferred embodiment of the method of the present invention;

[0063] Fig. 38 illustrates the purification of the conjugate HSA:fourth insulin derivative (SEQ ID NO:28) by a preferred embodiment of the method of the present invention;

[0064] Fig. 39 illustrates the purification of the conjugate HSA:first GRF analogue (SEQ ID NO:2) by a preferred embodiment of the method of the present invention;

[0065] Fig. 40 illustrates the purification of the conjugate HSA:second GRF analogue (SEQ ID NO:29) by a preferred embodiment of the method of the present invention;

[0066] Fig. 41 illustrates the purification of the conjugate HSA:third GRF analogue (SEQ ID NO:30) by a preferred embodiment of the method of the present invention;

[0067] Fig. 42 illustrates the purification of the conjugate HSA:fourth GRF analogue (SEQ ID NO:31) by a preferred embodiment of the method of the present invention;

[0068] Fig. 43 illustrates the purification of the conjugate HSA:thirteenth GLP-1 analogue CJC 1365 (SEQ ID NO:32) by a preferred embodiment of the method of the present invention;

[0069] Fig. 44 illustrates the purification of the conjugate HSA lactose:first GLP-1 analogue (SEQ ID NO:1) by a preferred embodiment of the method of the present invention;

[0070] Fig. 45 illustrates the purification of the conjugate HSA:first T20 analogue (SEQ ID NO:33) by a preferred embodiment of the method of the present invention;

[0071] Fig. 46 illustrates the purification of the conjugate HSA:first T1249 analogue (SEQ ID NO:34) by a preferred embodiment of the method of the present invention;

[0072] Fig. 47 illustrates the purification of the compound HSA:first GLP-1 analogue (SEQ ID NO:1) by a preferred embodiment of the method of the present invention;

[0073] Fig. 48 illustrates the purification of the compound HSA:first C34 analogue (SEQ ID NO:6) by a preferred embodiment of the method of the present invention;

[0074] Fig. 49 illustrates the purification of the compound HSA:second GRF analogue (SEQ ID NO:29) by a preferred embodiment of the method of the present invention;

[0075] Fig. 50 illustrates the purification of the conjugate HSA:vinorelbine analogue conjugate (SEQ ID NO:35) by a preferred embodiment of the method of the present invention;

[0076] Fig. 51 illustrates the purification of L-cysteine by a preferred embodiment of the method of the present invention;

[0077] Fig. 52 illustrates the purification of the conjugate L-Cysteine: vinorelbine analogue (SEQ ID NO:35) by a preferred embodiment of the method of the present invention;

[0078] Fig. 53 illustrates the purification of the conjugate RSA: third Exendin-4 analogue (SEQ ID NO:25) by a preferred embodiment of the method of the present invention;

[0079] Fig. 54 illustrates the purification of the conjugate HSA:fourth C34 analogue (SEQ ID NO:36) by a preferred embodiment of the method of the present invention;

[0080] Fig. 55 illustrates the purification of the conjugate HSA:fifth C34 analogue (SEQ ID NO:37) by a preferred embodiment of the method of the present invention;

[0081] Fig. 56 illustrates the purification of the conjugate HSA:sixth C34 analogue (SEQ ID NO:38) by a preferred embodiment of the method of the present invention;

[0082] Fig. 57 illustrates the purification of the conjugate HSA:seventh C34 analogue (SEQ ID NO:39) by a preferred embodiment of the method of the present invention;

[0083] Fig. 58 illustrates the purification of the conjugate HSA:eighth C34 analogue (SEQ ID NO:40) by a preferred embodiment of the method of the present invention;

[0084] Fig. 59 illustrates the purification of the conjugate HSA:first PYY analogue (SEQ ID NO:41) by a preferred embodiment of the method of the present invention;

[0085] Fig. 60 illustrates the purification of the conjugate HSA:second PYY analogue (SEQ ID NO:42) by a preferred embodiment of the method of the present invention;

[0086] Fig. 61 illustrates the purification of the conjugate HSA:fifth insulin derivative (SEQ ID NO:43) by a preferred embodiment of the method of the present invention;

[0087] Fig. 62 illustrates the purification of the conjugate HSA:sixth insulin derivative (SEQ ID NO:44) by a preferred embodiment of the method of the present invention;

[0088] Fig. 63 illustrates the purification of the conjugate HSA:seventh insulin derivative (SEQ ID NO:45) by a preferred embodiment of the method of the present invention;

[0089] Fig. 64 illustrates the purification of the conjugate HSA:third PYY analogue (SEQ ID NO:46) by a preferred embodiment of the method of the present invention;

[0090] Fig. 65 illustrates the purification of the conjugate HSA:fourth PYY analogue (SEQ ID NO:47) by a preferred embodiment of the method of the present invention;

[0091] Fig. 66 illustrates the purification of the conjugate HSA:fifth PYY analogue (SEQ ID NO:48) by a preferred embodiment of the method of the present invention;

[0092] Fig. 67 illustrates the purification of the conjugate HSA:sixth PYY analogue (SEQ ID NO:49) by a preferred embodiment of the method of the present invention;

[0093] Fig. 68 illustrates the purification of the conjugate HSA:second ANP analogue (SEQ ID NO:50) by a preferred embodiment of the method of the present invention;

[0094] Figs. 69A-B illustrates the purification of the conjugate HSA:third ANP analogue CJC 1681 (SEQ ID NO:51) by a preferred embodiment of the method of the present invention;

[0095] Fig. 70 illustrates the purification of the conjugate HSA:first GLP-1 analogue (SEQ ID NO:1) by a preferred embodiment of the method of the present invention;

[0096] Fig. 71 illustrates the purification of the conjugate HSA:first GLP-1 analogue (SEQ ID NO:1) by a preferred embodiment of the method of the present invention;

[0097] Fig. 72 illustrates the purification of the conjugate HSA:first GLP-1 analogue (SEQ ID NO:1) by a preferred embodiment of the method of the present invention;

[0098] Fig. 73 illustrates the purification of the conjugate HSA:first GLP-1 analogue (SEQ ID NO:1) by a preferred embodiment of the method of the present invention;

[0099] Fig. 74 illustrates the purification of the conjugate HSA:first GLP-1 analogue (SEQ ID NO:1) by a preferred embodiment of the method of the present invention;

[00100] Fig. 75 illustrates the purification of the conjugate HSA:first GLP-2 analogue (SEQ ID NO:52) by a preferred embodiment of the method of the present invention; and

[00101] Fig. 76 illustrates the purification of the conjugate RSA: first GLP-2 analogue (SEQ ID NO:52) by a preferred embodiment of the method of the present invention.

DETAILED DESCRIPTION OF A PREFERRED EMBODIMENT OF THE INVENTION

[00102] In accordance with the present invention, there is provided a method for purifying albumin conjugates from a solution comprising albumin conjugates and unconjugated albumin.

Methods

Preparation of control (non-conjugated) human serum albumin (HSA) and preformed albumin conjugates

[00103] Each compound with the Michael acceptor was solubilized in nanopure water (or in DMSO if the compound was difficult to solubilize) at a concentration of 10mM, then diluted to 1mM into a solution of HSA (25%, 250 mg/ml, Cortex-Biochem, San Leandro, CA). The samples were then incubated at 37°C for 30 min. Prior to their purification, each conjugate solution was diluted to 5% 50 mg/ml HSA in 20 mM sodium phosphate buffer (pH 7) composed of 5 mM sodium octanoate. The initial concentration of salt used in the elution gradient can be added to the buffer for diluting the mixed solution. Preferably, the initial concentration of salt is from about 750 to about 1700 mM $(NH_4)_2SO_4$.

Procedure for purification according to a preferred embodiment

[00104] Using an ÄKTA purifier (Amersham Biosciences, Uppsala, Sweden), each conjugate was loaded at a flow rate of 2.5 ml/min onto a 50 ml column of butyl sepharose 4 fast flow resin (Amershan Biosciences, Uppsala, Sweden) equilibrated in 20 mM sodium phosphate buffer (pH 7) composed of 5 mM sodium octanoate and 750 mM to 1.7 M $(NH_4)_2SO_4$. Under these

conditions, HSA conjugates having a molecular weight addition of more than 2 kDa relative to non-conjugated HSA adsorbed onto the hydrophobic resin whereas essentially all non-conjugated HSA eluted within the void volume of the column. For molecular weight additions of less than 2kDa, a higher initial salt content may be used followed by a stepwise gradient of decreasing salt. Each conjugate was further purified from any free unconjugated compound by applying a continuous or non-continuous decreasing gradient of salt (750 to 0 mM $(NH_4)_2SO_4$) over 4 column volumes. In a preferred embodiment, each purified conjugate was then desalted and concentrated by diafiltration, for instance by using Amicon® ultra centrifugal (30 kDa) filter devices (Millipore Corporation, Bedford, MA). Finally, for prolonged storage, each conjugate solution is preferably immersed into liquid nitrogen, and lyophilized using a Labconco freeze dry system (FreeZone®4.5), and stored at -20°C.

Examples of LC/EMS analysis

[00105] Following purification, 1 µl of each conjugate sample is preferably injected onto LC/EMS system. The HSA:first GLP-1 analogue (SEQ ID NO:1) conjugate was confirmed by detection of a species of highest abundance with a total mass of 70 160 Da which corresponds to the mass of mercaptalbumin (66 448 Da) where cysteine 34 is in the free thiol form, plus the mass of only one molecule of the first GLP-1 analogue (3 719.9 Da). The structure of the first GLP-1 analogue (SEQ ID NO:1) is described in Example 1 below. This is illustrated in Table 2.

Table 2

Component	Molecular Weight	Absolute Abundance	Relative Abundance
A	70160.58	321970	100.00
B	65862.95	70008	21.74
C	64545.45	62888	19.53
D	70320.04	41167	12.79

E	61287.67	16842	5.23
F	60623.81	16522	5.13
G	58090.04	12473	3.87

[00106] The HSA:first GRF analogue (SEQ ID NO:2) conjugate was confirmed by detection of a species of highest abundance with a total mass of 70 086 Da which corresponds to the mass of mercaptalbumin (66 448 Da) where cysteine 34 is in the free thiol form, plus the mass of only one molecule of the first GRF analogue (3648.2 Da). The structure of the first GRF analogue (SEQ ID NO:2) is described in Example 2 below. This is illustrated in Table 3.

Table 3

Component	Molecular Weight	Absolute Abundance	Relative Abundance
A	70086.06	279413	100.00
B	63214.84	53333	19.09
C	62148.17	38582	13.81
D	70247.98	34870	12.48
E	56795.96	10523	3.77
F	62695.49	9813	3.51

[00107] The following examples illustrate several compounds having a maleimide group as Michael acceptor that have been conjugated to albumin and purified in accordance with the method of the present invention.

[00108] The following examples are for the purpose of illustrating the present invention and not of limiting its scope.

[00109] In the following examples, the gradient numbers refer to the following gradient details, where CV means a column volume of 50 ml.

[00110] Gradient #1: Linear 750-0mM $(NH_4)_2SO_4$, over 4CV, flow rate of 2.5ml/min.

[00111] Gradient #2: Step gradient 1.75M-1.2M $(NH_4)_2SO_4$ over 0.5CV, followed by 1.2M-875mM $(NH_4)_2SO_4$ over 5CV, and finally 875mM-0mM $(NH_4)_2SO_4$ over 0.5CV flow rate of 2.5ml/min.

Gradient #3: Linear 900-0mM $(NH_4)_2SO_4$ over 4CV, flow rate of 2.5ml/min.

Gradient #4: Step gradient 1.5M-1.1M $(NH_4)_2SO_4$ over 0.5CV, followed by 1.1M-375mM $(NH_4)_2SO_4$ over 6CV, and finally 375mM-0mM $(NH_4)_2SO_4$ over 0.5CV, flow rate of 2.5ml/min.

Gradient #5: Linear 750-0mM $(NH_4)_2SO_4$ over 2CV, flow rate of 2.5ml/min.

Gradient #6: Step gradient 1.75M-0M $(NH_4)_2SO_4$ over 6CV, flow rate of 2.5ml/min.

Gradient #7: Linear 750-0mM $(NH_4)_2SO_4$ over 6CV, flow rate of 2.5ml/min.

Example 1

Purification of HSA:first GLP-1 analogue (SEQ ID NO:1) conjugate

[00112] The first GLP-1 analogue is GLP-1 (7-36) dAla^8 Lys^{37} (ε-AEEA-MPA)-$CONH_2$ and has the following sequence:

H(dA)EGTFTSDVSSYLEGQAAKEFIAWLVKGRK(AEEA-MPA)-$CONH_2$

[00113] The purification of a conjugate made from reacting 1 ml 25% 250mg/ml HSA (Cortex-Biochem, San Leandro, CA) with 1mM first GLP-1 analogue diluted into 9 ml of buffer made of 20 mM sodium phosphate buffer pH 7.0, 5 mM sodium caprylate and 750 mM $(NH_4)_2SO_4$, was performed on a column of Butyl sepharose using the gradient #1 described above. In Fig. 1 the purified conjugate fraction elutes during the gradient of decreasing $(NH_4)_2SO_4$ concentration as fraction B (F8-F9), whereas non-conjugated albumin elutes within the void volume of the column (fraction A). The conjugate fraction was concentrated with Ultrafree™ filter 30kDa and analyzed using LC-EMS.

Example 2

Purification of HSA:first GRF analogue (SEQ ID NO:2) conjugate

[00114] The first GRF analogue is GRF (1-29) dAla^2 Gln^8 Ala^{15} Leu^{27} Lys^{30} (ε-MPA) $CONH_2$ and has the following sequence:

YaDAIFTQSYRKVLAQLSARKLLQDILSRK(MPA)-$CONH_2$

[00115] The purification of a conjugate made from reacting 1 ml 25% 250mg/ml HSA (Cortex-Biochem, San Leandro, CA) with 1 mM first GRF analogue diluted into 9 ml of 20 mM sodium phosphate buffer (pH 7.0), 5 mM sodium caprylate and 750 mM $(NH_4)_2SO_4$, was performed on a column of Butyl sepharose using the gradient #1 described above. In Fig. 2 the purified conjugate fraction appears in fraction B (F6-F7) whereas non-conjugated albumin elutes within the void volume of the column (fraction A). The conjugate fraction was concentrated with Ultrafree™ filter 30kDa and analyzed using LC-EMS.

Example 3

Purification of non-conjugated HSA 1 ml

[00116] The purification of 1 ml 25% 250mg/ml non-conjugated HSA (Cortex-Biochem, San Leandro, CA) diluted into 9 ml of buffer (pH 7.0) made of 20 mM sodium phosphate buffer, 5 mM sodium caprylate and 750 mM $(NH_4)_2SO_4$, was performed on a column of Butyl sepharose using the gradient #1 described above. Essentially all albumin molecules elute within the void volume and no protein species is observed at 280nm during $(NH_4)_2SO_4$ gradient. Fig. 3 illustrates the separation curve obtained.

Example 4

Purification of rHSA:first GLP-1 analogue (SEQ ID NO:1) conjugate

[00117] The first GLP-1 analogue is GLP-1 (7-36) dAla^8 Lys^{37} (ε-AEEA-MPA)-$CONH_2$ and his sequence is shown in Example 1.

[00118] The purification of a conjugate made from reacting 5 ml 5% rHSA (recombinant HSA new century culture grade) with 200 µM first GLP-1 analogue diluted into 5 ml of a buffer made of 20 mM sodium phosphate buffer, 5 mM sodium caprylate and 750 mM $(NH_4)_2SO_4$ was performed on a column of Butyl sepharose using the gradient #1 described above. In Fig. 4 the purified conjugate fraction appears in fraction B (F7-F8-F9).

Example 5

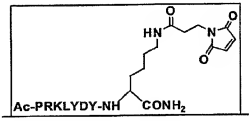
Purification of HSA 10 ml

[00119] The purification of 10 ml 25% 250mg/ml HSA (Cortex-Biochem, San Leandro, CA) diluted into 40 ml of a buffer made of 20 mM sodium phosphate buffer (pH 7.0), 5 mM sodium caprylate and 750 mM $(NH_4)_2SO_4$ was performed on a column of Butyl sepharose using the gradient #1 described above. Essentially all albumin molecules elute within a void volume and no protein species is observed at 280nm during $(NH_4)_2SO_4$ gradient. Fig. 5 illustrates the separation curve obtained.

Example 6

Purification of HSA:K5 analogue (SEQ ID NO:3) conjugate

[00120] The K5 analogue is Ac-K5 Lys^8 (ε-MPA)-NH_2 and has the following sequence:



[00121] The purification of a conjugate made from reacting 4 ml 25% 250 mg/ml HSA (Cortex-Biochem, San Leandro, CA) with 1 mM K5 analogue diluted into 16 ml of 20 mM sodium phosphate buffer (pH 7.0), 5 mM sodium

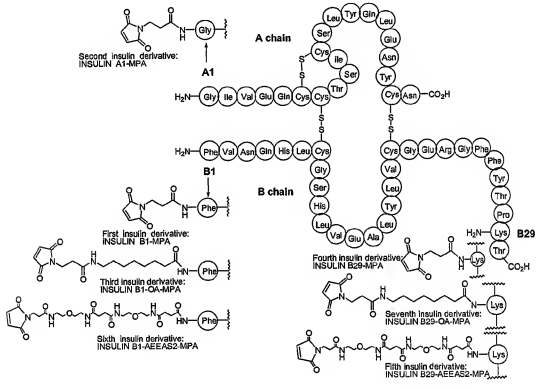
caprylate and 750 mM $(NH_4)_2SO_4$ was performed on a column of Butyl sepharose using the gradient #1 described above. In Fig. 6 the purified conjugate fraction appears in fraction A with albumin and in fraction B (F6-F7-F8).

Example 7

Purification of HSA:first insulin derivative (SEQ ID NO:4) conjugate

[00122] The first insulin derivative is human insulin with MPA on position B1 and is represented in Figure 1 below.

Figure 1



[00123] The purification of a conjugate made from reacting 1 ml 25% 250mg/ml HSA (Cortex-Biochem, San Leandro, CA) with 1 mM first insulin derivative diluted into 9 ml of 20 mM sodium phosphate buffer (pH 7.0), 5 mM sodium caprylate and 750 mM $(NH_4)_2SO_4$ was performed on a column of

Butyl sepharose using the gradient #1 described above. In Fig. 7 the purified conjugate fraction appears in fraction B (F6-F7-F8).

Example 8

Purification of HSA:second insulin derivative (SEQ ID NO:5) conjugate

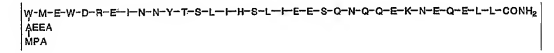
[00124] The second insulin derivative is human insulin with MPA on position A1 and is represented in Figure 1 shown above in Example 7.

[00125] The purification of a conjugate made from reacting 1 ml 25% 250mg/ml HSA (Cortex-Biochem, San Leandro, CA) with 1 mM second insulin derivative diluted into 9 ml of 20 mM sodium phosphate buffer (pH 7.0), 5 mM sodium caprylate and 750 mM $(NH_4)_2SO_4$ was performed on a column of Butyl sepharose using gradient #1 described above. In Fig. 8 the purified conjugate fraction appears in fraction B (F6-F7-F8).

Example 9

Purification of HSA:first C34 analogue (SEQ ID NO:6) conjugate

[00126] The first C34 analogue is MPA-AEEA-C34-$CONH_2$ and has the following sequence:

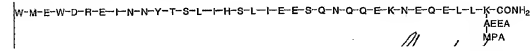


[00127] The purification of a conjugate made from reacting 5 ml 25% 250mg/ml HSA (Cortex-Biochem, San Leandro, CA) with 1 mM first C34 analogue diluted into 20 ml of 20 mM sodium phosphate buffer (pH 7.0), 5 mM sodium caprylate and 750 mM $(NH_4)_2SO_4$ was performed on a column of Butyl sepharose using gradient #1 described above. In Fig. 9 the purified conjugate fraction appears in fraction F2.

Example 10

Purification of HSA:second C34 analogue (SEQ ID NO:7) conjugate

[00128] The second C34 analogue is C34 (1-34) Lys^{35} (ε-AEEA-MPA)-$CONH_2$ and has the following structure:

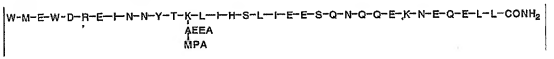


[00129] The purification of a conjugate made from reacting 5 ml 25% 250mg/ml HSA (Cortex-Biochem, San Leandro, CA) with 1 mM second C34 analogue diluted into 20 ml of 20 mM sodium phosphate buffer, 5 mM sodium caprylate and 750 mM $(NH_4)_2SO_4$ was performed on a column of Butyl sepharose using gradient #1 described above. In Fig. 10 the purified conjugate fraction appears in fraction F2.

Example 11

Purification of HSA:third C34 analogue (SEQ ID NO:8) conjugate

[00130] The third C34 analogue is C34 (1-34) Lys^{13} (ε-AEEA-MPA)-$CONH_2$ and has the following structure:



[00131] The purification of a conjugate made from reacting 5 ml 25% 250mg/ml HSA (Cortex-Biochem, San Leandro, CA) with 1 mM third C34 analogue diluted into 20 ml of 20 mM sodium phosphate buffer (pH 7.0), 5 mM sodium caprylate and 750 mM $(NH_4)_2SO_4$, was performed on a column of Butyl sepharose using gradient #1 described above. In Fig. 11 the purified conjugate fraction appears in fraction F2.

Example 12

Purification of l-cysteine

[00132] The purification of 121mg of l-cysteine in 2 ml of a buffer made of 20 mM sodium phosphate, 5 mM sodium caprylate and 750 mM $(NH_4)_2SO_4$, was performed on a column of Butyl sepharose using the gradient #5 described above. Fig. 12 illustrates the separation curve obtained, where L-cysteine elutes within the void volume of the column (F3).

Example 13

Purification of L-cysteine:first GLP-1 analogue (SEQ ID NO:1) conjugate

[00133] The first GLP-1 analogue is GLP-1 (7-36) $dAla^8$ Lys^{37} (ε-AEEA-MPA)-$CONH_2$ and his sequence is shown above in Example 1.

[00134] The purification of a conjugate made from reacting 121 mg L-cysteine with 36.36 mg first GLP-1 analogue diluted into 2 ml of 20 mM sodium phosphate buffer (pH 7.0), 5 mM sodium caprylate and 750 mM $(NH_4)_2SO_4$ was performed on a column of Butyl sepharose using gradient #5 described above. Fig. 13 illustrates the separation curve obtained where the excess L-cysteine elutes in F3 (column void volume) and the L-Cysteine:first GLP-1 analogue conjugate elutes in 0mM $(NH_4)_2SO_4$.

Example 14

Purification of HSA:second GLP-1 analogue (SEQ ID NO:9) conjugate

[00135] The second GLP-1 analogue is GLP-1 (7-36) Lys^{37} (ε-MPA)-NH_2 and has the following sequence:

HAEGTFTSDVSSYLEGQAAKEFIAWLVKGRK(ε-MPA)

[00136] The purification of a conjugate made from reacting 2.5 ml 25% 250mg/ml HSA (Cortex-Biochem, San Leandro, CA) with 1 mM second GLP-1 analogue diluted into 10 ml of 20 mM sodium phosphate buffer (pH 7.0), 5 mM sodium caprylate and 750 mM $(NH_4)_2SO_4$ was performed on a

column of Butyl sepharose using gradient #5 described above. In Fig. 14 the purified conjugate fraction appears in fraction F2.

Example 15

Purification of HSA:third GLP-1 analogue (SEQ ID NO:10) conjugate

[00137] The third GLP-1 analogue is GLP-1 (7-36) dAla^{8} Lys^{37} (ε-MPA)-NH_2 and has the following sequence:

H(dA)EGTFTSDVSSYLEGQAAKEFIAWLVKGRK(MPA)-$CONH_2$

[00138] The purification of a conjugate made from reacting 2.5 ml 25% 250mg/ml HSA (Cortex-Biochem, San Leandro, CA) with 1 mM third GLP-1 analogue diluted into 10 ml of 20 mM sodium phosphate buffer (pH 7.0), 5 mM sodium caprylate and 750 mM $(NH_4)_2SO_4$ was performed on a column of Butyl sepharose using gradient #5 described above. In Fig. 15 the purified conjugate fraction appears in fraction F2.

Example 16

Purification of HSA:fourth GLP-1 analogue (SEQ ID NO:11) conjugate

[00139] The fourth GLP-1 analogue is GLP-1 (7-36) Lys^{26} (ε-AEEA-AEEA-MPA) and has the following sequence:

HAEGTFTSDVSSYLEGQAAK(ε-AEEA-AEEA-MPA) EFIAWLVKGR

[00140] The purification of a conjugate made from reacting 2.5 ml 25% 250mg/ml HSA (Cortex-Biochem, San Leandro, CA) with 1 mM fourth GLP-1 analogue diluted into 10 ml of 20 mM sodium phosphate buffer (pH 7.0), 5 mM sodium caprylate and 750 mM $(NH_4)_2SO_4$ was performed on a column of Butyl sepharose using gradient #1 described above. In Fig. 16 the purified conjugate fraction appears in fraction F2.

Example 17

Purification of HSA:fifth GLP-1 analogue (SEQ ID NO:12) conjugate

[00141] The fifth GLP-1 analogue is GLP-1 (7-36) Lys^{34} (ε-AEEA-AEEA-MPA)and has the following sequence:

HAEGTFTSDVSSYLEGQAAKEFIAWLVK(ε-AEEA-AEEA-MPA)GR

[00142] The purification of a conjugate made from reacting 2.5 ml 25% 250mg/ml HSA (Cortex-Biochem, San Leandro, CA) with 1 mM fifth GLP-1 analogue diluted into 10 ml of 20 mM sodium phosphate buffer (pH 7.0), 5 mM sodium caprylate and 750 mM $(NH_4)_2SO_4$ was performed on a column of Butyl sepharose using gradient #1 described above. In Fig. 17 the purified conjugate fraction appears in fraction F2.

Example 18

Purification of HSA:first Exendin-4 analogue (SEQ ID NO:13) conjugate

[00143] The first exendin-4 analogue is Exendin-4-(1-39) Lys^{40} (ε-MPA)-NH_2 and has the following sequence:

HGEGTFTSDLSKQMEEEAVRLFIEWLKNGGPSSGAPPPSK(ε-MPA)-$CONH_2$

[00144] The purification of a conjugate made from reacting 1 ml 25% 250mg/ml HSA (Cortex-Biochem, San Leandro, CA) with 1 mM first Exendin-4 analogue diluted into 9 ml of 20 mM sodium phosphate buffer (pH 7.0), 5 mM sodium caprylate and 750 mM $(NH_4)_2SO_4$ was performed on a column of Butyl sepharose using gradient #1 described above. In Fig. 18 the purified conjugate fraction appears in fraction F2.

Example 19

Purification of HSA:second Exendin-4 analogue (SEQ ID NO:14) conjugate

[00145] The second Exendin-4 analogue is Exendin-4 (9-39) Lys^{40} (ε-AEEA-MPA)-$CONH_2$ and has the following sequence:

DLSKQMEEEAVRLFIEWLKNGGPSSGAPPPSK(AEEA-MPA)-$CONH_2$

[00146] The purification of a conjugate made from reacting 3.5 ml 25% HSA cortex with 1 mM second Exendin-4 analogue diluted into 21.5 ml of 20 mM sodium phosphate buffer (pH 7.0), 5 mM sodium caprylate and 750 mM $(NH_4)_2SO_4$ was performed on a column of Butyl sepharose using gradient #1 described above. In Fig. 19 the purified conjugate fraction appears in fraction F2.

Example 20

Purification of HSA:MPA

[00147] The purification of a conjugate made from reacting 1 ml 25% 250mg/ml HSA (Cortex-Biochem, San Leandro, CA) with 2 mM MPA diluted into 9 ml of 20 mM sodium phosphate buffer (pH 7.0), 5 mM sodium caprylate and 1750 mM $(NH_4)_2SO_4$ was performed on a column of Butyl sepharose using gradient #2 described above. In Fig. 20 the fraction of mercaptalbumin is in fraction A (F5) and capped albumin is in fraction B (F7-F8). The conjugate fraction was concentrated with Amicon™ filter 30kDa.

Example 21

Purification of HSA

[00148] The purification of 1 ml 25% 250mg/ml HSA (Cortex-Biochem, San Leandro, CA) diluted into 9 ml of 20 mM sodium phosphate buffer (pH 7.0), 5 mM sodium caprylate and 1750 mM $(NH_4)_2SO_4$ was performed on a column of Butyl sepharose using the gradient #2 described above. When using gradient #2, unlike gradients #1 and #5, both conjugated albumin and non-conjugated albumin adsorbs onto the hydrophobic resin during sample loading. Fig. 21 illustrates the separation curve obtained where F4 and F5 are enriched in mercaptalbumin and F6, F7 and F8 are enriched in capped albumin.

Example 22

Purification of HSA: second C34 analogue (SEQ ID NO:3) conjugate

[00149] The second C34 analogue is C34 (1-34) Lys^{35} (ε-AEEA-MPA)-$CONH_2$ and his structure is shown in Example 10.

[00150] The purification of a conjugate made from reacting 1 ml 25% 250mg/ml HSA (Cortex-Biochem, San Leandro, CA) with 1mM second C34 analogue diluted into 9 ml of a buffer made of 20 mM sodium phosphate buffer (pH 7.0), 5 mM sodium caprylate and 1750 mM $(NH_4)_2SO_4$ was performed on a column of Butyl sepharose using gradient #2 described above. In Fig. 22 mercaptalbumin appears in fraction A (F5) and capped albumin and the purified conjugated is in fraction B (F7-F8).

Example 23

Purification of HSA : first Dynorphin A analogue (SEQ ID NO:15) conjugate

[00151] The first Dynorphin A analogue is Dyn A (1-13) (MPA)-NH_2 and has the following sequence: **YGGFLRRIRPKLK(MPA)-$CONH_2$.**

[00152] The purification of a conjugate made from reacting 1 ml 25% 250mg/ml HSA (Cortex-Biochem, San Leandro, CA) with 1 mM first Dynorphin A analogue diluted into 9 ml of 20 mM sodium phosphate buffer (pH 7.0), 5 mM sodium caprylate and 1750 mM $(NH_4)_2SO_4$ was performed on a column of Butyl sepharose using gradient #2 described above. In Fig. 23 the purified conjugate fraction appears in fraction A (F11-F12)

Example 24

Purification of HSA :first ANP analogue (SEQ ID NO:16) conjugate

[00153] The first ANP analogue is MPA-AEEA-ANP (99-126)-$CONH_2$ and has the following structure:

MPA-AEEA-Ser-Leu-Arg-Arg-Ser-Ser-Cys-Phe-Gly-Gly-Arg-Met-Asp-
|
Arg-Ile-Gly-Ala-Gln-Ser-Gly-Leu-Gly-Cys-Asn-Ser-Phe-Arg-Tyr-$CONH_2$

[00154] The purification of a conjugate made from reacting 1 ml 25% 250mg/ml HSA (Cortex-Biochem, San Leandro, CA) with 1 mM first ANP analogue diluted into 9 ml of 20 mM sodium phosphate buffer (pH 7.0), 5 mM sodium caprylate and 1750 mM $(NH_4)_2SO_4$ was performed on a column of Butyl sepharose using gradient #2 described above. In Fig. 24 the purified conjugate fraction appears in fraction A (F14).

Example 25

Purification of HSA:second Dynorphin A analogue (SEQ ID NO:17) conjugate

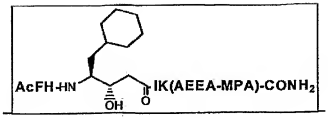
[00155] The second Dynorphin A analogue is Dyn A (7-13) Lys^{13} (ε-MPA)-$CONH_2$ and has the following sequence : **RIRPKLK(MPA)-$CONH_2$**

[00156] The purification of a conjugate made from reacting 1 ml 25% 250mg/ml HSA (Cortex-Biochem, San Leandro, CA) with 1 mM second Dynorphin A analogue diluted into 9 ml of 20 mM sodium phosphate buffer (pH 7.0), 5 mM sodium caprylate and 1750 mM $(NH_4)_2SO_4$ was performed on a column of Butyl sepharose using gradient #2 described above. In Fig. 25 the purified conjugate fraction appears in fraction A (F9).

Example 26

Purification of HSA:ACE inhibitor (SEQ ID NO:18) conjugate

[00157] The ACE inhibitor used in this example is acetyl-Phe-His-cyclohexylstatyl-Ile-Lys (ε-AEEA-MPA)-$CONH_2$ and has the following sequence:



[00158] The purification of a conjugate made from reacting 1 ml 25% 250mg/ml HSA (Cortex-Biochem, San Leandro, CA) with 1 mM ACE inhibitor diluted into 9 ml of 20 mM sodium phosphate buffer (pH 7.0), 5 mM sodium caprylate and 1750 mM $(NH_4)_2SO_4$ was performed on a column of Butyl sepharose using gradient #2 described above. In Fig. 26 the purified conjugate fraction appears in fraction A (F14).

Example 27

Purification of HSA : sixth GLP-1 analogue (SEQ ID NO:19) conjugate

[00159] The sixth GLP-1 analogue is GLP-1 (7-36) Lys^{23} (ε-AEEA-MPA)-$CONH_2$ and has the following sequence:

HAEGTFTSDVSSYLEGK(AEEA-MPA)AAKEFIAWLVKGR-$CONH_2$

[00160] The purification of a conjugate made from reacting 3 ml 25% 250mg/ml HSA (Cortex-Biochem, San Leandro, CA) with 1 mM sixth GLP-1 analogue diluted into 22 ml of 20 mM sodium phosphate buffer (pH 7.0), 5 mM sodium caprylate and 1750 mM $(NH_4)_2SO_4$ was performed on a column of Butyl sepharose using gradient #1 described above. In Fig. 27 the purified conjugate fraction appears in fraction F2.

Example 28

Purification of HSA:seventh GLP-1 analogue (SEQ ID NO:20) conjugate

[00161] The seventh GLP-1 analogue is GLP-1 (7-36) Lys^{18} (ε-AEEA-MPA)-$CONH_2$ and has the following sequence:

HAEGTFTSDVSK(AEEA-MPA)YLEGQAAKEFIAWLVKGR-CONH$_2$

[00162] The purification of a conjugate made from reacting 3 ml 25% 250mg/ml HSA (Cortex-Biochem, San Leandro, CA) with 1 mM seventh GLP-1 analogue diluted into 22 ml of 20 mM sodium phosphate buffer (pH 7.0), 5 mM sodium caprylate and 750 mM $(NH_4)_2SO_4$ was performed on a column of Butyl sepharose using gradient #1 described above. In Fig. 28 the purified conjugate fraction appears in fraction F2.

Example 29

Purification of HSA: eighth GLP-1 analogue (SEQ ID NO:21) conjugate

[00163] The eighth GLP-1 analogue is GLP-1 (7-36) Lys^{26} (ε-AEEA-MPA)-$CONH_2$ and has the following sequence:

HAEGTFTSDVSSYLEGQAAK(AEEA-MPA)EFIAWLVKGR-CONH$_2$

[00164] The purification of a conjugate made from reacting 2.5 ml 25% 250mg/ml HSA (Cortex-Biochem, San Leandro, CA) with 1 mM eighth GLP-1 analogue diluted into 22.5 ml of 20 mM sodium phosphate buffer (pH 7.0), 5 mM sodium caprylate and 750 mM $(NH_4)_2SO_4$ was performed on a column of Butyl sepharose using gradient #1 described above. In Fig. 29 the purified conjugate fraction appears in fraction F2.

Example 30

Purification of HSA:ninth GLP-1 analogue (SEQ ID NO:22) conjugate

[00165] The ninth GLP-1 analogue is GLP-1 (7-37) Lys^{27} (ε-AEEA-MPA)-$CONH_2$ and has the following sequence:

HAEGTFTSDVSSYLEGQAAKK(AEEA-MPA)FIAWLVKGR-CONH$_2$

[00166] The purification of a conjugate made from reacting 3 ml 25% 250mg/ml HSA (Cortex-Biochem, San Leandro, CA) with 1 mM ninth GLP-1

analogue diluted into 22 ml of 20 mM sodium phosphate buffer (pH 7.0), 5 mM sodium caprylate and 750 mM $(NH_4)_2SO_4$ was performed on a column of Butyl sepharose using gradient #1 described above. In Fig. 30 the purified conjugate fraction appears in fraction F2.

Example 31

Purification of HSA:tenth GLP-1 analogue (SEQ ID NO:23) conjugate

[00167] The tenth GLP-1 analogue is GLP-1 (7-36) Lys^{37} (ε-AEEA-AEEA-MPA)-$CONH_2$ and has the following sequence:

[00168] **HAEGTFTSDVSSYLEGQAAKEFIAWLVKGRK-AEEA-AEEA-MPA-$CONH_2$**

[00169] The purification of a conjugate made from reacting 2.5 ml 25% 250mg/ml HSA (Cortex-Biochem, San Leandro, CA) with 1 mM tenth GLP-1 analogue diluted into 22.5 ml of 20 mM sodium phosphate buffer (pH 7.0), 5 mM sodium caprylate and 750 mM $(NH_4)_2SO_4$ was performed on a column of Butyl sepharose using gradient #1 described above. In Fig. 31 the purified conjugate fraction appears in fraction F2.

Example 32

Purification of HSA:eleventh GLP-1 analogue (SEQ ID NO:24) conjugate

[00170] The eleventh GLP-1 analogue is GLP-1 (7-36) Lys^{37} (ε-AEEA-MPA)-$CONH_2$ and has the following sequence:

HAEGTFTSDVSSYLEGQAAKEFIAWLVKGRK(AEEA-MPA)-$CONH_2$

[00171] The purification of a conjugate made from reacting 2.5 ml 25% 250mg/ml HSA (Cortex-Biochem, San Leandro, CA) with 1 mM eleventh GLP-1 analogue diluted into 22.5 ml of 20 mM sodium phosphate buffer (pH 7.0), 5 mM sodium caprylate and 750 mM $(NH_4)_2SO_4$ was performed on a column of Butyl sepharose using gradient #1 described above. In Fig. 32 the purified conjugate fraction appears in fraction F2.

Example 33

Purification of HSA:third Exendin-4 analogue (SEQ ID NO:25) conjugate

[00172] The third Exendin-4 analogue is Exendin-4-(1-39) Lys^{40} (ε-AEEA-MPA)-$CONH_2$ and has the following sequence:

HGEGTFTSDLSKQMEEEAVRLFIEWLKNGGPSSGAPPPSK(ε-AEEA-MPA)-$CONH_2$

[00173] The purification of a conjugate made from reacting 2.5 ml 25% 250mg/ml HSA (Cortex-Biochem, San Leandro, CA) with 1 mM third Exendin-4 analogue diluted into 22.5 ml of 20 mM sodium phosphate buffer (pH 7.0), 5 mM sodium caprylate and 750 mM $(NH_4)_2SO_4$ was performed on a column of Butyl sepharose using gradient #1 described above. In Fig. 33 the purified conjugate fraction appears in fraction F2.

Example 34

Purification of HSA:twelfth GLP-1 analogue (SEQ ID NO:26) conjugate

[00174] The twelfth GLP-1 analogue is GLP-1 (7-36) Lys^{34} (ε-AEEA-MPA)-$CONH_2$ and has the following sequence:

HAEGTFTSDVSSYLEGQAAKEFIAWLVK(ε-AEEA-MPA)GR-$CONH_2$

[00175] The purification of a conjugate made from reacting 2.5 ml 25% 250mg/ml HSA (Cortex-Biochem, San Leandro, CA) with 1 mM twelfth GLP-1 analogue diluted into 22.5 ml of 20 mM sodium phosphate buffer (pH 7.0), 5 mM sodium caprylate and 750 mM $(NH_4)_2SO_4$ was performed on a column of Butyl sepharose using gradient #1 described above. In Fig. 34 the purified conjugate fraction appears in fraction F2.

Example 35

Purification of HSA:first insulin derivative (SEQ ID NO:4) conjugate

[00176] The first insulin derivative is human insulin with MPA on position B1 and his structure is detailed in Example 7.

[00177] The purification of a conjugate made from reacting 2.5 ml 25% 250mg/ml HSA (Cortex-Biochem, San Leandro, CA) with 1 mM first insulin derivative diluted into 22.5 ml of 20 mM sodium phosphate buffer (pH 7.0), 5 mM sodium caprylate and 750 mM $(NH_4)_2SO_4$ was performed on a column of Butyl sepharose using gradient #1 described above. In Fig. 35 the purified conjugate fraction appears in fraction F2.

Example 36

Purification of HSA:third insulin derivative (SEQ ID NO:27) conjugate

[00178] The third insulin derivative is human insulin with OA-MPA on position B1 and is represented in Figure 1 shown above in Example 7.

[00179] The purification of a conjugate made from reacting 4 ml 25% 250mg/ml HSA (Cortex-Biochem, San Leandro, CA) with 1mM third insulin derivative diluted into 21 ml of 20 mM sodium phosphate buffer (pH 7.0), 5 mM sodium caprylate and 750 mM $(NH_4)_2SO_4$ was performed on a column of Butyl sepharose using gradient #1 described above. In Fig. 36 the purified conjugate fraction appears in fraction F2.

Example 37

Purification of HSA:second insulin derivative (SEQ ID NO:5) conjugate

[00180] The second insulin derivative is human insulin with MPA on position A1 and is represented in Figure 1 shown above in Example 7.

[00181] The purification of a conjugate made from reacting 3 ml 25% 250mg/ml HSA (Cortex-Biochem, San Leandro, CA) with 1mM second insulin derivative diluted into 22 ml of 20 mM sodium phosphate buffer (pH 7.0), 5 mM sodium caprylate and 750 mM $(NH_4)_2SO_4$ was performed on a column

of Butyl sepharose using gradient #1 described above. In Fig. 37 the purified conjugate fraction appears in fraction F2.

Example 38

Purification of HSA:fourth insulin derivative (SEQ ID NO:28) conjugate

[00182] The fourth insulin derivative is human insulin with MPA on position B29 and is represented in Figure 1 shown above in Example 7.

[00183] The purification of a conjugate made from reacting 3 ml 25% 250mg/ml HSA (Cortex-Biochem, San Leandro, CA) with 1mM fourth insulin derivative diluted into 22 ml of 20 mM sodium phosphate buffer (pH 7.0), 5 mM sodium caprylate and 750 mM $(NH_4)_2SO_4$ was performed on a column of Butyl sepharose using gradient #1 described above. In Fig. 38 the purified conjugate fraction appears in fraction F2.

Example 39

Purification of HSA:first GRF analogue (SEQ ID NO:2) conjugate

[00184] The first GRF analogue is GRF (1-29) $dAla^2$ Gln^8 Ala^{15} Leu^{27} Lys^{30} (ε-MPA) $CONH_2$ and his sequence is shown in Example 2.

[00185] The purification of a conjugate made from reacting 3.7 ml 25% 250mg/ml HSA (Cortex-Biochem, San Leandro, CA) with 1 mM first GRF analogue diluted into 22 ml of 20 mM sodium phosphate buffer (pH 7.0), 5 mM sodium caprylate and 750 mM $(NH_4)_2SO_4$ was performed on a column of Butyl sepharose using gradient #1 described above. In Fig. 39 the purified conjugate fraction appears in fraction F2.

Example 40

Purification of HSA:second GRF analogue (SEQ ID NO:29) conjugate

[00186] The second GRF analogue is GRF(1-29) Lys^{30} (ε-MPA)-$CONH_2$ and has the following sequence:

YADAIFTNSYRKVLGQLSARKLLQDIMSRK(MPA)-CONH$_2$

[00187] The purification of a conjugate made from reacting 2.5 ml 25% 250mg/ml HSA (Cortex-Biochem, San Leandro, CA) with 1 mM second GRF analogue diluted into 22.5 ml of 20 mM sodium phosphate buffer (pH 7.0), 5 mM sodium caprylate and 900 mM $(NH_4)_2SO_4$ was performed on a column of Butyl sepharose using gradient #3 described above. In Fig. 40 the purified conjugate fraction appears in fraction F2.

Example 41

Purification of HSA:third GRF analogue (SEQ ID NO:30) conjugate

[00188] The third GRF analogue is GRF (1-29) dAla^2 Gln^8 dArg^{11} Ala^{15} Leu^{27} Lys^{30} (ε-MPA)-CONH$_2$ and has the following sequence:

YaDAIFTQSYrKVLAQLSARKLLQDILSRK(MPA)-CONH$_2$

[00189] The purification of a conjugate made from reacting 2.5 ml 25% 250mg/ml HSA (Cortex-Biochem, San Leandro, CA) with 1mM third GRF analogue diluted into 22.5 ml of 20 mM sodium phosphate buffer (pH 7.0), 5 mM sodium caprylate and 750 mM $(NH_4)_2SO_4$ was performed on a column of Butyl sepharose using gradient #3 described above. In Fig. 41 the purified conjugate fraction appears in fraction F2.

Example 42

Purification of HSA:fourth GRF analogue (SEQ ID NO:31) conjugate

[00190] The fourth GRF analogue is GRF (1-29) dAla^2 Lys^{30} (ε-MPA)-CONH$_2$ and has the following sequence:

YaDAIFTNSYRKVLGQLSARKLLQDIMSRK(MPA)-CONH$_2$

[00191] The purification of a conjugate made from reacting 2.5 ml 25% HSA (Cortex-Biochem, San Leandro, CA) with 1mM fourth GRF analogue

diluted in 22.5 ml of 20 mM sodium phosphate buffer (pH 7.0), 5 mM sodium caprylate and 900 mM $(NH_4)_2SO_4$ was performed on a column of Butyl sepharose using gradient #3 described above. In Fig. 42 the purified conjugate fraction appears in fraction F2.

Example 43

Purification of HSA: thirteenth GLP-1 analogue CJC 1365 (SEQ ID NO:32) conjugate

[00192] The thirteenth GLP-1 analogue is GLP-1 (9-36) Lys^{37} (ε-AEEA-MPA)-$CONH_2$ and has the following sequence:

EGTFTSDVSSYLEGQAAKEFIAWLVKGRK(ε-AEEA-MPA)-$CONH_2$

[00193] The purification of a conjugate made from reacting 3.5 ml 25% HSA (Cortex-Biochem, San Leandro, CA) and 1mM thirteenth GLP-1 analogue diluted in 21.5 ml of 20 mM sodium phosphate buffer (pH 7.0), 5 mM sodium caprylate and 750 mM $(NH_4)_2SO_4$ was performed on a column of Butyl sepharose using gradient #1 described above. In Fig. 43 the purified conjugate fraction appears in fraction F2.

Example 44

Purification of HSA lactose:first GLP-1 analogue (SEQ ID NO :1) conjugate

[00194] The first GLP-1 analogue is GLP-1 (7-36) $dAla^8$ Lys^{37} (ε-AEEA-MPA)-$CONH_2$ and his sequence is shown above in Example 1.

[00195] The purification of a conjugate made from reacting 4 ml 25% lactosaminated albumin (HSA pre-incubated with excess lactose at 37°C, pH 7.0) with 200μM first GLP-1 analogue in 4 ml of a buffer made of 20 mM sodium phosphate, 5 mM sodium caprylate and 750 mM $(NH_4)_2SO_4$, (pH 7.0) was performed on a column of Butyl sepharose using gradient #1 described above. In Fig. 44 the purified lactosaminated conjugate fraction appears in fraction F2.

Example 45

Purification of HSA:first T20 analogue (SEQ ID NO:33) conjugate

[00196] The first T20 analogue is Ac-T20 (1-36) Lys^{37} (ε-AEEA-MPA)-$CONH_2$ and ahs the following sequence:

Ac-YTSLIHSLIEESQNQQEKNEQELLELDKWASLWNWFK(AEEA-MPA)-$CONH_2$

[00197] The purification of a conjugate made from reacting 2.5 ml 25% HSA with 1 mM first T20 analogue in 10 ml of 20 mM sodium phosphate buffer (pH 7.0), 5 mM sodium caprylate and 750 mM $(NH_4)_2SO_4$ was performed on a column of Butyl sepharose using gradient #1 described above. In Fig. 45 the purified conjugate fraction appears in fraction F2.

Example 46

Purification of HSA: first T1249 analogue (SEQ ID NO:34) conjugate

[00198] The first T1249 analogue is Ac-T1249 (1-39) Lys^{40} (ε-AEEA-MPA)-$CONH_2$ and has the following sequence:

Ac-WQEWEQKITALLEQAQIQQEKNEYELQKLDKWASLWEWFK(AEEA-MPA)-$CONH_2$

[00199] The purification of a conjugate made from reacting 2 ml 25% HSA and 1 mM first T1249 analogue in 10.5 ml of 20 mM sodium phosphate buffer (pH 7.0), 5 mM sodium caprylate and 750 mM $(NH_4)_2SO_4$ was performed on a column of Butyl sepharose using gradient #1 described above. In Fig. 46 the purified conjugate fraction appears in fraction F4.

Example 47

Purification of a HSA: first GLP-1 analogue (SEQ ID NO:1)

[00200] The first GLP-1 analogue is GLP-1 (7-36) $dAla^8$ Lys^{37} (ε-AEEA-MPA)-$CONH_2$ and his sequence is shown in Example 1.

[00201] The purification of 114.45mg of the preformed conjugate of the first GLP-1 analogue in 12.5 ml of 20 mM sodium phosphate buffer (pH 7.0), 5 mM

sodium caprylate and 750 mM $(NH_4)_2SO_4$ was performed on a column of Butyl sepharose using gradient #5 described above. Fig. 47 illustrates the separation curve obtained with the conjugate found in fraction F2.

Example 48

Purification of a HSA:first C34 analogue (SEQ ID NO:6)

[00202] The first C34 analogue is MPA-AEEA-C34-$CONH_2$ and his sequence is shown above in Example 9.

[00203] The purification of 114.45mg of the preformed conjugate of the first C34 analogue in 12.5 ml of 20 mM sodium phosphate buffer (pH 7.0), 5 mM sodium caprylate and 750 mM $(NH_4)_2SO_4$ was performed on a column of Butyl sepharose using gradient #5 described above. Fig. 48 illustrates the separation curve obtained with the conjugate found in fraction F2.

Example 49

Purification of a HSA:second GRF analogue (SEQ ID NO:29)

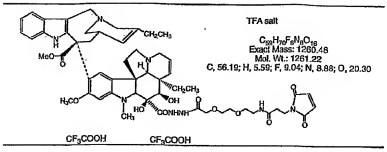
[00204] The second GRF analogue is GRF(1-29) Lys^{30} (ε-MPA)-$CONH_2$ and his sequence is shown above in Example 40.

[00205] The purification of 125.53mg of the preformed conjugate of the second GRF analogue in 12.5 ml of 20 mM sodium phosphate buffer (pH 7.0), 5 mM sodium caprylate and 750 mM $(NH_4)_2SO_4$, pH 7.0 was performed on a column of Butyl sepharose using gradient #5 described above. Fig. 49 illustrates the separation curve obtained with the conjugate found in fraction F2.

Example 50

Purification of HSA:vinorelbine analogue conjugate (SEQ ID NO:35)

[00206] The vinorelbine analogue is a molecule of vinorelbine with AEEA-MPA coupled thereto as illustrated in the following structure:



[00207] The purification of a conjugate made from 2.5 ml 25% HSA and 1mM vinorelbine analogue in 22.5ml of a buffer made of 20 mM sodium phosphate buffer, 5 mM sodium caprylate and 750 mM $(NH_4)_2SO_4$, pH 7.0 was performed on a column of Butyl sepharose using gradient #4 described above. In Fig. 50 the purified conjugate fraction appears in fraction F2. The conjugate fraction was concentrated with Amicon™ filter 30kDa.

Example 51

Purification of L-Cysteine

[00208] The purification of 2.5 ml 40mM L-cysteine in 22.5 ml of 20 mM sodium phosphate buffer (pH 7.0), 5 mM sodium caprylate and 1500 mM $(NH_4)_2SO_4$, was performed on a column of Butyl sepharose using gradient #4 described above. Fig. 51 illustrates the separation curve obtained with L-cysteine eluting within the void volume of the column (fraction F3).

Example 52

Purification of L-Cysteine: vinorelbine analogue (SEQ ID NO:35) conjugate

[00209] The vinorelbine analogue is a molecule of vinorelbine with AEEA-MPA coupled thereto as illustrated in the structure shown in Example 50.

[00210] The purification of a conjugate made from reacting 2.5 ml 40mM L-cysteine with 1mM vinorelbine analogue in 22.5 ml of 20 mM sodium phosphate buffer (pH 7.0), 5 mM sodium caprylate and 750 mM $(NH_4)_2SO_4$

was performed on a column of Butyl sepharose using gradient #4 described above. Fig. 52 illustrates the separation curve obtained with the L-cysteine conjugate eluting within fractions F8, F9 and F10.

Example 53

Purification of RSA: third Exendin-4 analogue (SEQ ID NO:25) conjugate

[00211] The third Exendin-4 analogue is Exendin-4-(1-39) Lys^{40} (ε-AEEA-MPA)-$CONH_2$ and his sequence shown in Example 33.

[00212] The purification of a conjugate made from reacting 11 ml 5% RSA (rat serum albumin) with 200μM third Exendin-4 analogue in 11 ml of 20 mM sodium phosphate buffer (pH 7.0), 5 mM sodium caprylate and 750 mM $(NH_4)_2SO_4$, was performed on a column of Butyl sepharose using gradient #1 described above. In Fig. 53 the purified conjugate fraction appears in fraction F2.

Example 54

Purification of HSA:fourth C34 analogue (SEQ ID NO:36) conjugate

[00213] The fourth C34 analogue is C34 (1-34) Lys^{13} (ε-MPA)-$CONH_2$ and has the following sequence:

WMEWDREINNYTK(MPA)LIHSLIEESQNQQEKNEQELL-$CONH_2$

[00214] The purification of a conjugate made from reacting 2 ml 25% HSA with 1mM fourth C34 analogue in 13 ml of 20 mM sodium phosphate buffer (pH 7.0), 5 mM sodium caprylate and 750 mM $(NH_4)_2SO_4$ was performed on a column of Butyl sepharose using gradient #1 described above. In Fig. 54 the purified conjugate fraction appears in fraction F2. .

Example 55

Purification of HSA:fifth C34 analogue (SEQ ID NO:37) conjugate

[00215] The fifth C34 analogue is C34 (1-34) Lys^{35} (ε-MPA)-$CONH_2$ and has the following sequence:

WMEWDREINNYTSLIHSLIEESQNQQEKNEQELLK(MPA)-$CONH_2$

[00216] The purification of a conjugate made from 2 ml 25% HSA and 1mM fifth C34 analogue in 13 ml of a buffer made of 20 mM sodium phosphate buffer (pH 7.0), 5 mM sodium caprylate and 750 mM $(NH_4)_2SO_4$, was performed on a column of Butyl sepharose using gradient #1 described above. In Fig. 55 the purified conjugate fraction appears in fraction F2.

Example 56

Purification of HSA:sixth C34 analogue (SEQ ID NO:38) conjugate

[00217] The sixth C34 analogue MPA-C34 (1-34)-$CONH_2$ and has the following sequence:

MPA-WMEWDREINNYTSLIHSLIEESQNQQEKNEQELL-$CONH_2$

[00218] The purification of a conjugate made from reacting 2 ml 25% HSA and 1mM sixth C34 analogue in 13 ml of 20 mM sodium phosphate buffer (pH 7.0), 5 mM sodium caprylate and 750 mM $(NH_4)_2SO_4$ was performed on a column of Butyl sepharose using gradient #1 described above. In Fig. 56 the purified conjugate fraction appears in fraction F2.

Example 57

Purification of HSA:seventh C34 analogue (SEQ ID NO:39) conjugate

[00219] The seventh C34 analogue is Ac-C34 (1-34) Glu^2 Lys^6 Lys^7 Glu^9 Glu^{10} Lys^{13} Lys^{14} Glu^{16} Glu^{17} Lys^{20} Lys^{21} Glu^{23} Glu^{24} Lys^{27} Glu^{31} Lys^{34} Lys^{35} Lys^{36} (ε-AEEA-MPA)-$CONH_2$ and has the following sequence:

Ac-WEEWDKKIEEYTKKIEELIKKSEEQQKKNEEELKKK(AEEA-MPA)-$CONH_2$

[00220] The purification of a conjugate made from reacting 2 ml 25% HSA with 1mM seventh C34 analogue in 13 ml 20 mM sodium phosphate buffer (pH 7.0), 5 mM sodium caprylate and 750 mM $(NH_4)_2SO_4$, was performed on a

column of Butyl sepharose using gradient #1 described above. In Fig. 57 the purified conjugate fraction appears in fraction F2.

Example 58

Purification of HSA:eighth C34 analogue (SEQ ID NO:40) conjugate

[00221] The eighth C34 analogue is MPA-AEEA-C34 (1-34) Glu^2 Lys^6 Lys^7 Glu^9 Glu^{10} Lys^{13} Lys^{14} Glu^{16} Glu^{17} Lys^{20} Lys^{21} Glu^{23} Glu^{24} Lys^{27} Glu^{31} Lys^{34} Lys^{35} $^*CONH_2$ and has the following sequence:

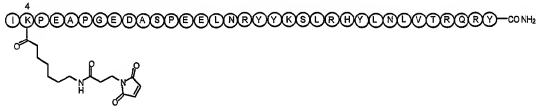
MPA-AEEA-WEEWDKKIEEYTKKIEELIKKSEEQQKKNEEELKK-$CONH_2$

[00222] The purification of a conjugate made from reacting 2 ml 25% HSA with 1mM eighth C34 analogue in 13 ml of 20 mM sodium phosphate buffer (pH 7.0), 5 mM sodium caprylate and 750 mM $(NH_4)_2SO_4$, was performed on a column of Butyl sepharose using gradient #1 described above. In Fig. 58 the purified conjugate fraction appears in fraction F2.

Example 59

Purification of HSA:first PYY analogue (SEQ ID NO:41) conjugate

[00223] The first PYY analogue is PYY (3-36) Lys^4 (ε-OA-MPA)-$CONH_2$ and has the following structure:

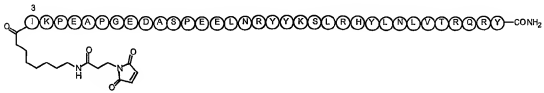


[00224] The purification of a conjugate made from reacting 1.5 ml 25% HSA with 1mM first PYY analogue in 6 ml of 20 mM sodium phosphate buffer (pH 7.0), 5 mM sodium caprylate and 750 mM $(NH_4)_2SO_4$, was performed on a column of Butyl sepharose using gradient #1 described above. In Fig. 59 the purified conjugate fraction appears in fraction F2.

Example 60

Purification of HSA:second PYY analogue (SEQ ID NO:42) conjugate

[00225] The second PYY analogue is MPA-OA-PYY (3-36)-$CONH_2$ and has the following sequence:



[00226] The purification of a conjugate made from reacting 1.5 ml 25% HSA with 1mM second PYY analogue in 6 ml of 20 mM sodium phosphate buffer (pH 7.0), 5 mM sodium caprylate and 750 mM $(NH_4)_2SO_4$, was performed on a column of Butyl sepharose using gradient #1 described above. In Fig. 60 the purified conjugate fraction appears in fraction F2.

Example 61

Purification of HSA:fifth insulin derivative (SEQ ID NO:43) conjugate

[00227] The fifth insulin derivative is human insulin with AEEAS-AEEAS-MPA on position B29 and is represented in Figure 1 shown above in Example 7.

[00228] The purification of a conjugate made from reacting 2 ml 25% HSA with 1mM fifth insulin derivative in 15 ml of 20 mM sodium phosphate buffer (pH 7.0), 5 mM sodium caprylate and 750 mM $(NH_4)_2SO_4$ was performed on a column of Butyl sepharose using gradient #1 described above. In Fig. 61 the purified conjugate fraction appears in fraction F2.

Example 62

Purification of HSA:sixth insulin derivative (SEQ ID NO:44) conjugate

[00229] The sixth insulin derivative is human insulin with AEEAS-AEEAS-MPA on position B1 and is represented in Figure 1 shown above in Example 7.

[00230] The purification of a conjugate made from reacting 2.5 ml 25% HSA with 1mM sixth insulin derivative in 15 ml of 20 mM sodium phosphate buffer (pH 7.0), 5 mM sodium caprylate and 750 mM $(NH_4)_2SO_4$ was performed on a column of Butyl sepharose using gradient #1 described above. In Fig. 62 the purified conjugate fraction appears in fraction F2.

Example 63

Purification of HSA:seventh insulin derivative (SEQ ID NO:45) conjugate

[00231] The seventh insulin derivative is human insulin with OA-MPA on position B29 and is represented in Figure 1 shown above in Example 7.

[00232] The purification of a conjugate made from reacting 2 ml 25% HSA with 1mM seventh insulin derivative in 15 ml of 20 mM sodium phosphate buffer (pH 7.0), 5 mM sodium caprylate and 750 mM $(NH_4)_2SO_4$ was performed on a column of Butyl sepharose using gradient #1 described above. In Fig. 63 the purified conjugate fraction appears in fraction F2.

Example 64

Purification of HSA:third PYY analogue (SEQ ID NO:46) conjugate

[00233] The third PYY analogue is MPA-PYY (3-36)-$CONH_2$ and has the following sequence:

MPA-NH-IKPEAPGEDASPEELNRYYASLRHYLNLVTRQRY-$CONH_2$

[00234] The purification of a conjugate made from reacting 3 ml 25% HSA with 1mM third PYY analogue in 18 ml of 20 mM sodium phosphate buffer (pH 7.0), 5 mM sodium caprylate and 750 mM $(NH_4)_2SO_4$ was performed on a column of Butyl sepharose using gradient #1 described above. In Fig. 64 the purified conjugate fraction appears in fraction F2.

Example 65

Purification of HSA:fourth PYY analogue (SEQ ID NO:47) conjugate

[00235] The fourth PYY analogue is PYY (3-36) Lys^{37} (ε-MPA)-$CONH_2$ and has the following sequence:

[00236] **IKPEAPGEDASPEELNRYYASLRHYLNLVTRQRYK(MPA)-$CONH_2$**

[00237] The purification of a conjugate made from reacting 3 ml 25% HSA with 1mM fourth PYY analogue in 18 ml of 20 mM sodium phosphate buffer (pH 7.0), 5 mM sodium caprylate and 750 mM $(NH_4)_2SO_4$ was performed on a column of Butyl sepharose using gradient #1 described above. In Fig. 65 the purified conjugate fraction appears in fraction F2.

Example 66

Purification of HSA:fifth PYY analogue (SEQ ID NO:48) conjugate

[00238] The fifth PYY analogue is MPA-PYY (22-36)-$CONH_2$ and has the following sequence: **(MPA)-ASLRHYLNLVTRQRY-$CONH_2$.**

[00239] The purification of a conjugate made from reacting 6 ml 25% HSA with 1mM fifth PYY analogue in 36 ml of 20 mM sodium phosphate buffer (pH 7.0), 5 mM sodium caprylate and 900 mM $(NH_4)_2SO_4$ was performed on a column of Butyl sepharose using gradient #3 described above. In Fig. 66 the purified conjugate fraction appears in fraction F2.

Example 67

Purification of HSA:sixth PYY analogue (SEQ ID NO:49) conjugate

[00240] The sixth PYY analogue is Acetyl-PYY (22-36) Lys^{37} (ε-MPA)-$CONH_2$ and has the following sequence: **Ac-ASLRHYLNLVTRQRYK(MPA)-$CONH_2$.**

[00241] The purification of a conjugate made from reacting 6 ml 25% HSA with 1mM sixth PYY analogue in 36 ml of 20 mM sodium phosphate buffer (pH 7.0), 5 mM sodium caprylate and 900 mM $(NH_4)_2SO_4$ was performed on a column of Butyl sepharose using gradient #3 described above. In Fig. 67 the purified conjugate fraction appears in fraction F2.

Example 68

Purification of HSA:second ANP analogue (SEQ ID NO:50) conjugate

[00242] The second ANP analogue is MPA-ANP (99-126)-$CONH_2$ and has the following structure:

MPA-Ser-Leu-Arg-Arg-Ser-Ser-Cys-Phe-Gly-Gly-Arg-Met-Asp-Arg-
|
Ile-Gly-Ala-Gln-Ser-Gly-Leu-Gly-Cys-Asn-Ser-Phe-Arg-Tyr-$CONH_2$

[00243] The purification of a conjugate made from reacting 1 ml 25% HSA with 1mM second ANP analogue in 14 ml of 20 mM sodium phosphate buffer (pH 7.0), 5 mM sodium caprylate and 750 mM $(NH_4)_2SO_4$ was performed on a column of Butyl sepharose using gradient #3 described above. In Fig. 68 the purified conjugate fraction appears in fraction F2.

Example 69

Purification of HSA:third ANP analogue (SEQ ID NO:51) conjugate

[00244] The third ANP analogue is ANP (99-126) having reacted with MAL-$dPEG_4$™ (Quanta Biodesign, Powell, OH, USA) coupled to Ser^{99}. The resulting ANP analogue is MPA-EEEEP-ANP (99-126) where EEEEP is ethoxy ethoxy ethoxy ethoxy propionic acid; and its sequence is the following:

MPA-EEEEP-Ser-Leu-Arg-Arg-Ser-Ser-Cys-Phe-Gly-Gly-Arg-Met-Asp-Arg-
|
Ile-Gly-Ala-Gln-Ser-Gly-Leu-Gly-Cys-Asn-Ser-Phe-Arg-Tyr-$CONH_2$

The purification of a conjugate made from reacting 1 ml 25% HSA with 1 mM CJC 1681 in 14 ml of 20 mM sodium phosphate buffer (pH 7.0), 5 mM sodium caprylate and 900 mM $(NH_4)_2SO_4$ was performed on a column of Butyl sepharose using gradient #3 described above. In Figs. 69A and 69B the purified conjugate fraction appears in fraction F2.

Example 70

Purification of HSA:first GLP-1 analogue (SEQ ID NO:1) conjugate

[00245] The first GLP-1 analogue is GLP-1 (7-36) dAla^{8} Lys^{37} (ε-AEEA-MPA)-$CONH_2$ and his sequence is shown above in Example 1.

[00246] The purification of a conjugate made from reacting 1 ml 25% 250mg/ml HSA (Cortex-Biochem, San Leandro, CA) with 1mM first GLP-1 analogue diluted into 9 ml of buffer made of 20 mM sodium phosphate buffer pH 7.0, 5 mM sodium caprylate and 1.75M $(NH_4)_2SO_4$, was performed on a column of Butyl sepharose using the gradient #6 described above. In Fig. 70 the purified conjugate fraction appears in fraction B.

Example 71

Purification of HSA: first GLP-1 analogue (SEQ ID NO:1) conjugate

[00247] The first GLP-1 analogue is GLP-1 (7-36) dAla^{8} Lys^{37} (ε-AEEA-MPA)-$CONH_2$ and his sequence is shown above in Example 1.

[00248] The purification of a conjugate made from reacting 1 ml 25% 250mg/ml HSA (Cortex-Biochem, San Leandro, CA) with 1mM first GLP-1 analogue diluted into 9 ml of buffer made of 20 mM sodium phosphate buffer pH 7.0, 5 mM sodium caprylate and 1.75M magnesium sulfate, was performed on a column of Butyl sepharose using the gradient #6 described above. In Fig. 71 the purified conjugate fraction appears in fraction F2.

Example 72

Purification of HSA: first GLP-1 analogue (SEQ ID NO:1) conjugate

[00249] The first GLP-1 analogue is GLP-1 (7-36) dAla^{8} Lys^{37} (ε-AEEA-MPA)-$CONH_2$ and his sequence is shown above in Example 1.

[00250] Example with 750mM ammonium sulfate The purification of a conjugate made from reacting 1 ml 25% 250mg/ml HSA (Cortex-Biochem, San Leandro, CA) with 1mM first GLP-1 analogue diluted into 9 ml of buffer made of 20 mM sodium phosphate buffer pH 7.0, 5 mM sodium caprylate and 750mM $(NH_4)_2SO_4$, was performed on a column of Butyl sepharose

using the gradient #1 described above. In Fig. 72 the purified conjugate fraction appears in fraction F2.

Example 73

Purification of HSA: first GLP-1 analogue (SEQ ID NO:1) conjugate

[00251] The first GLP-1 analogue is GLP-1 (7-36) dAla^8 Lys^{37} (ε-AEEA-MPA)-$CONH_2$ and his sequence is shown above in Example 1.

[00252] Example with 1.75M ammonium phosphate The purification of a conjugate made from reacting 1 ml 25% 250mg/ml HSA (Cortex-Biochem, San Leandro, CA) with 1mM first GLP-1 analogue diluted into 9 ml of buffer made of 20 mM sodium phosphate buffer pH 7.0, 5 mM sodium caprylate and 1.75M ammonium phosphate, was performed on a column of Butyl sepharose using the gradient #6 described above. In Fig. 73 the purified conjugate fraction appears in fraction B.

Example 74

Purification of HSA: first GLP-1 analogue (SEQ ID NO:1) conjugate

[00253] The first GLP-1 analogue is GLP-1 (7-36) dAla^8 Lys^{37} (ε-AEEA-MPA)-$CONH_2$ and his sequence is shown above in Example 1.

[00254] Example with 750mM ammonium phosphate The purification of a conjugate made from reacting 1 ml 25% 250mg/ml HSA (Cortex-Biochem, San Leandro, CA) with 1mM first GLP-1 analogue diluted into 9 ml of buffer made of 20 mM sodium phosphate buffer pH 7.0, 5 mM sodium caprylate and 750mM ammonium phosphate, was performed on a column of Butyl sepharose using the gradient #1 described above. In Fig. 74 the purified conjugate fraction appears in fraction F2.

Example 75

Purification of HSA: first GLP-2 analogue (SEQ ID NO:52) conjugate

[00255] The first GLP-2 analogue is GLP-2 (1-33) Gly^2 Lys^{34} (ε-MPA)-$CONH_2$ and has the following sequence:

HGDGSFSDEMNTILDNLAARDFINWLIQTKITDK(MPA)-CONH$_2$

[00256] The purification of a conjugate made from reacting 2 ml 25% 250mg/ml HSA (Cortex-Biochem, San Leandro, CA) with 1mM first GLP-2 analogue diluted into 14 ml of buffer made of 20 mM sodium phosphate buffer pH 7.0, 5 mM sodium caprylate and 750 mM $(NH_4)_2SO_4$, was performed on a column of Butyl sepharose using the gradient #1 described above. In Fig. 75 the purified conjugate fraction appears in fraction F2.

Example 76

Purification of RSA: first GLP-2 analogue (SEQ ID NO:52) conjugate

[00257] The first GLP-2 analogue is GLP-2 (1-33) Gly^2 Lys^{34} (ε-MPA)-$CONH_2$ and his sequence is shown in Example 75.

[00258] The purification of a conjugate made from reacting 9 ml 25% 250mg/ml RSA (rat serum albumin) with 1mM first GLP-2 analogue diluted into 14 ml of buffer made of 20 mM sodium phosphate buffer pH 7.0, 5 mM sodium caprylate and 750 mM $(NH_4)_2SO_4$, was performed on a column of Butyl sepharose using the gradient #1 described above. In Fig. 76 the purified conjugate fraction appears in fraction F2.

[00259] While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A method for separating albumin conjugate from unconjugated albumin in a solution comprising albumin conjugate and unconjugated albumin, the method comprising:

a) loading said solution onto a hydrophobic solid support equilibrated in aqueous buffer having a high salt content;

b) applying to said support a gradient of decreasing salt content; and

c) collecting eluted albumin conjugate.

2. The method of claim 1, wherein said albumin conjugate consists of a molecule having a Michael acceptor covalently coupled thereto which bonds to albumin.

3. The method of claim 2, wherein said bond is between said Michael acceptor and cysteine 34 of said albumin.

4. The method of claim 2, wherein said Michael acceptor is a maleimide group.

5. The method of claim 4, wherein said maleimide group is maleimid-propionic acid (MPA).

6. The method of claim 1, wherein said albumin is selected from the group consisting of serum albumin, recombinant albumin and albumin from a genomic source.

7. The method of claim 1, wherein said albumin is selected from the group consisting of human albumin, rat albumin, mouse albumin, swine albumin, bovine albumin, dog albumin and rabbit albumin.

8. The method of claim 1, wherein said albumin is human serum albumin.

9. The method of claim 1, wherein said albumin is modified with at least one selected from the group consisting of fatty acids, metal ions, small molecules having high affinity to albumin, and sugars.

10. The method of claim 9, wherein said sugars are selected from the group consisting of glucose, lactose and mannose.

11. The method of claim 2, wherein said molecule is selected from the group consisting of a peptide, DNA, RNA, small organic molecule and a combination thereof.

12. The method of claim 11, wherein said peptide has a molecular weight of at least 57 daltons.

13. The method of claim 11, wherein said peptide is selected from the group consisting of GLP-1, ANP, K5, dynorphin, GRF, insulin, natriuretic peptides, T-20, T-1249, C-34, SC-35, PYY and analogs thereof.

14. The method of claim 11, wherein said small organic molecule is selected from the group consisting of vinorelbine, gemcitabine and paclitaxel.

15. The method of claim 11, wherein said molecule is covalently attached to said albumin through an acid sensitive covalent bond or a peptide sequence susceptible to proteolytic cleavage, thereby allowing the separation of said molecule and albumin and the entry of the molecule into a cell.

16. The method of claim 1, wherein said hydrophobic solid support is a column containing a hydrophobic resin.

17. The method of claim 16, wherein said hydrophobic resin is selected from the group consisting of octyl sepharose, phenyl sepharose and butyl sepharose.

18. The method of claim 16, wherein said hydrophobic resin is butyl sepharose.

19. The method of claim 1, wherein said salt has a sufficent salting out effect.

20. The method of claim 1, wherein said salt is selected from the group consisting of ammonium phosphate, ammonium sulfate and magnesium phosphate.

21. The method of claim 1, wherein said salt is ammonium phosphate or ammonium sulfate.

22. The method of claim 1, wherein said salt is ammonium sulfate.

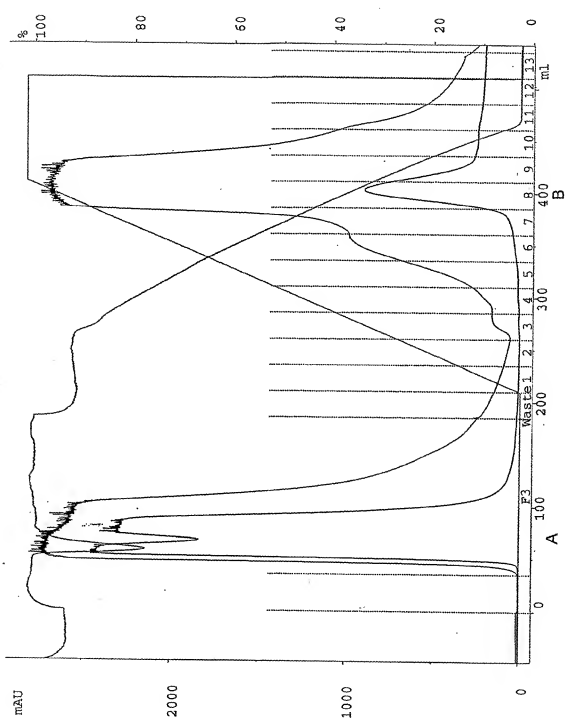

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Figure 1

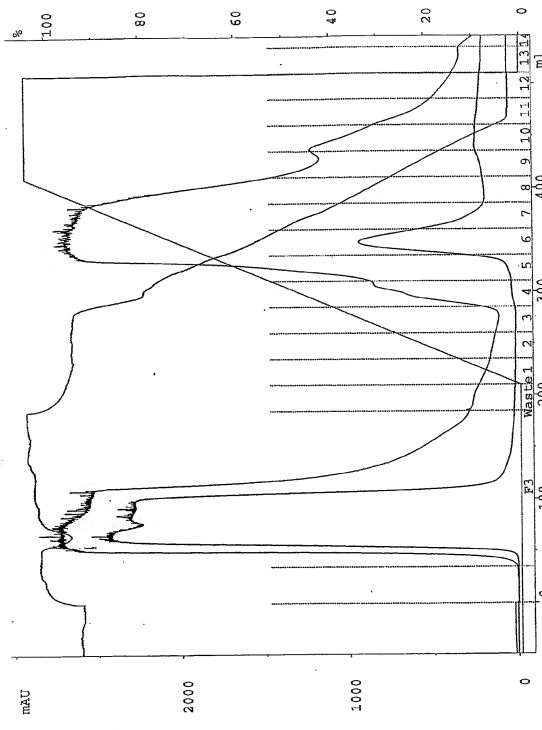
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Figure 2

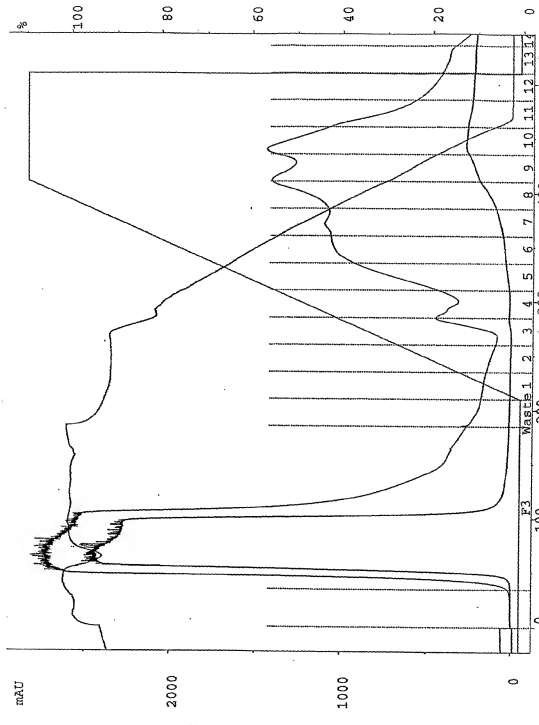
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Figure 3

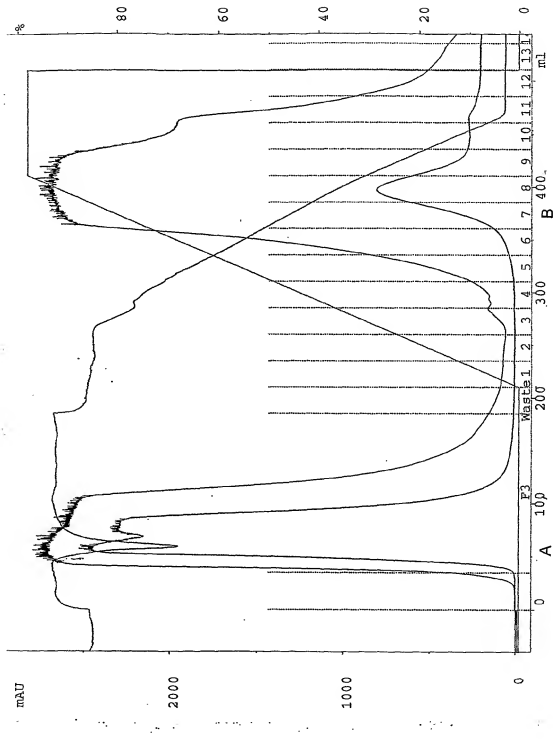
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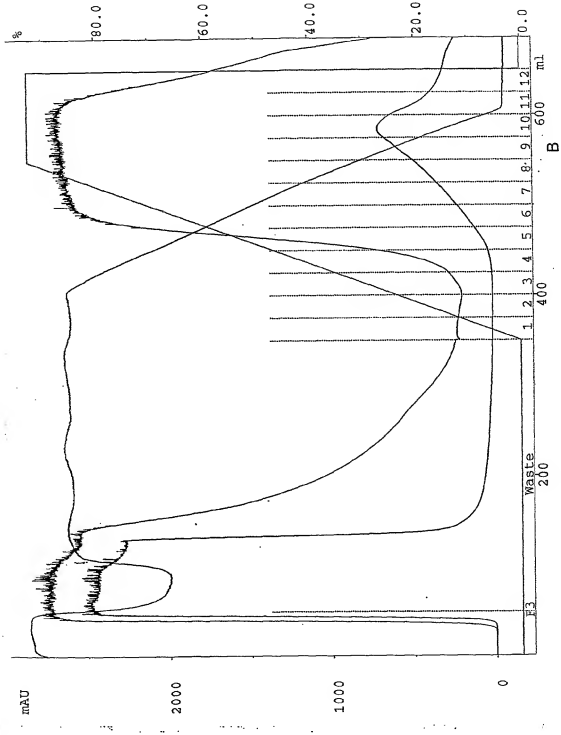
Figure 4



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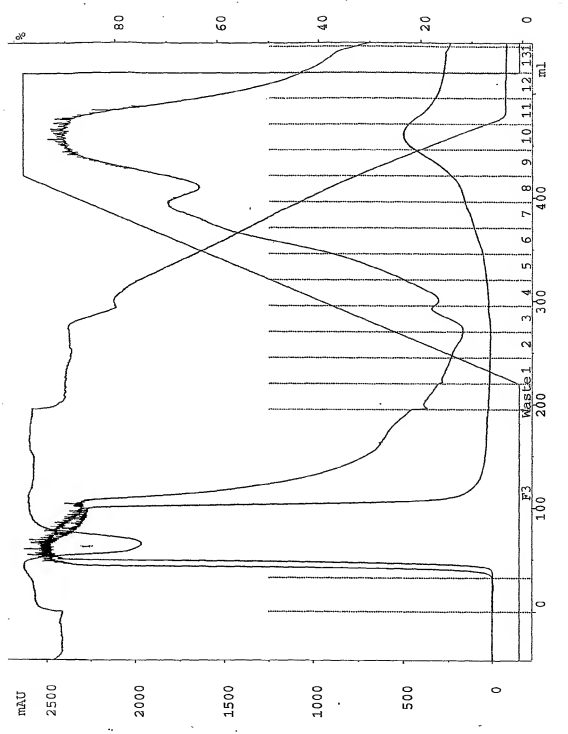
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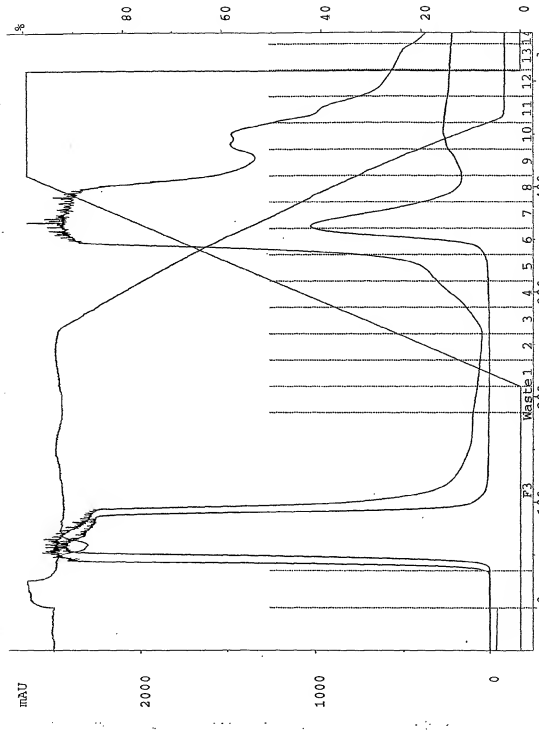
Figure 6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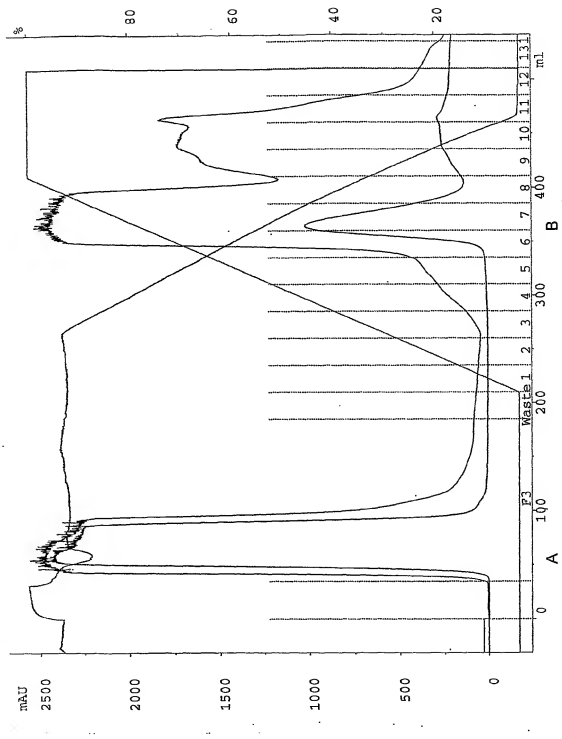
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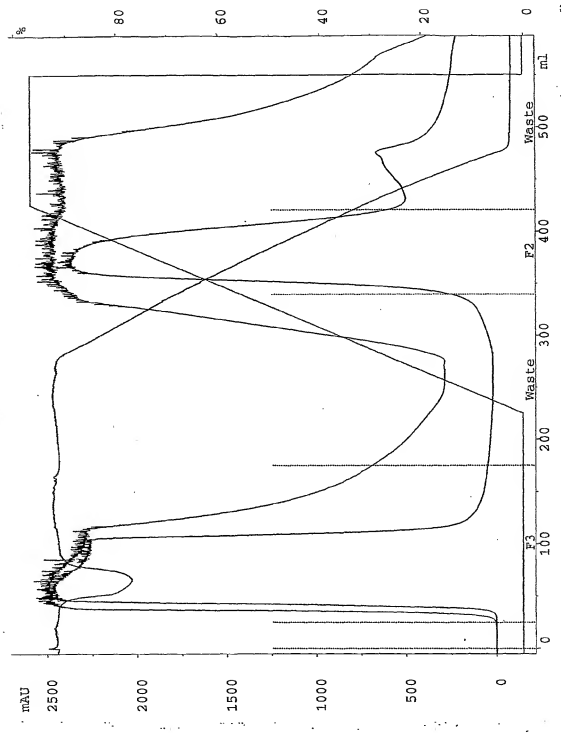
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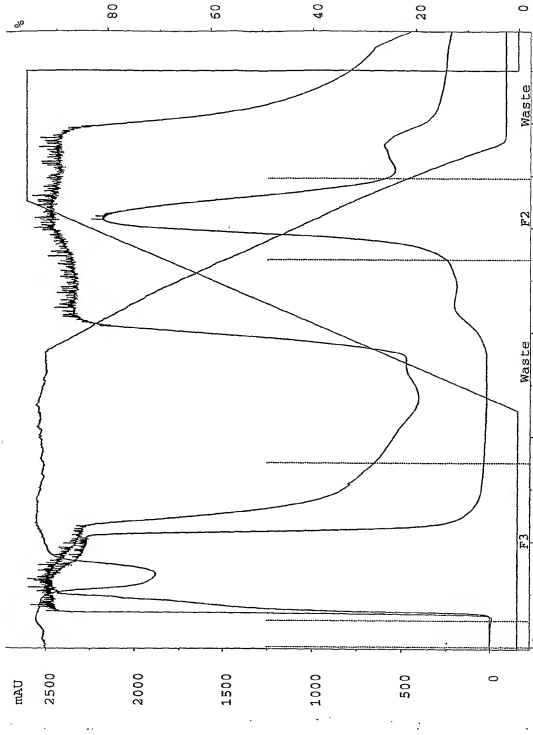
Figure 9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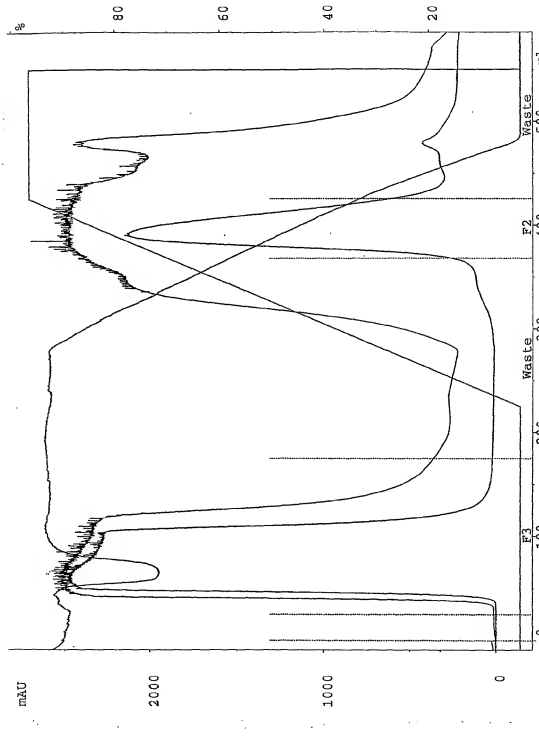
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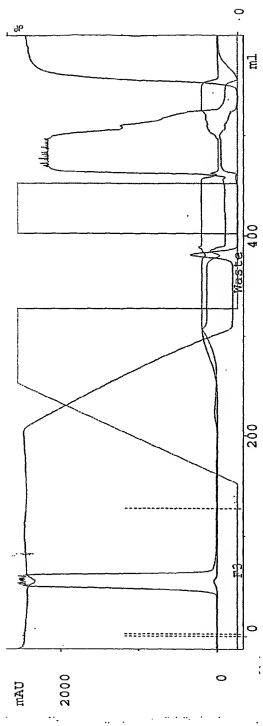
Figure 11



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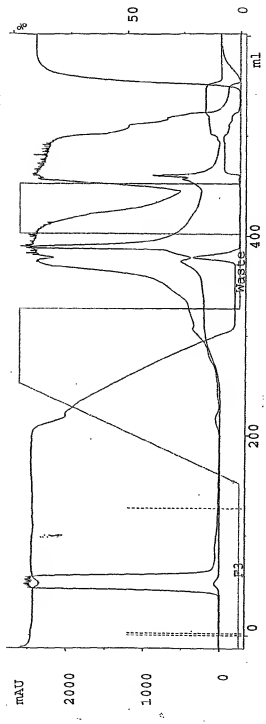

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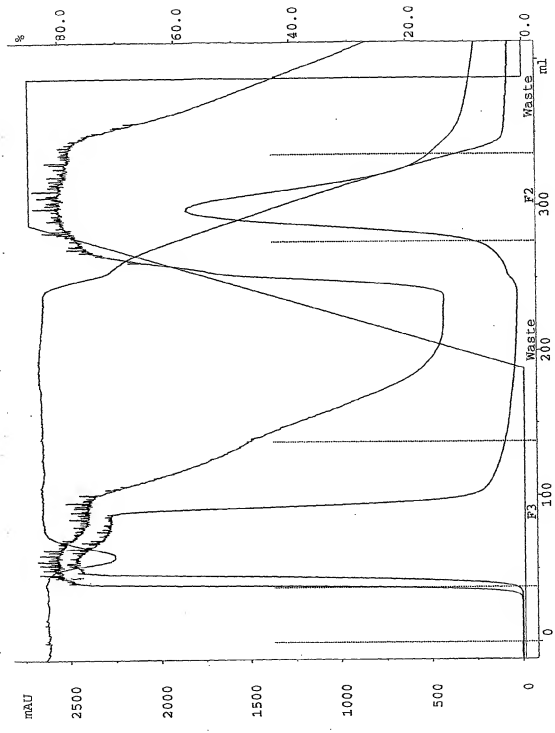
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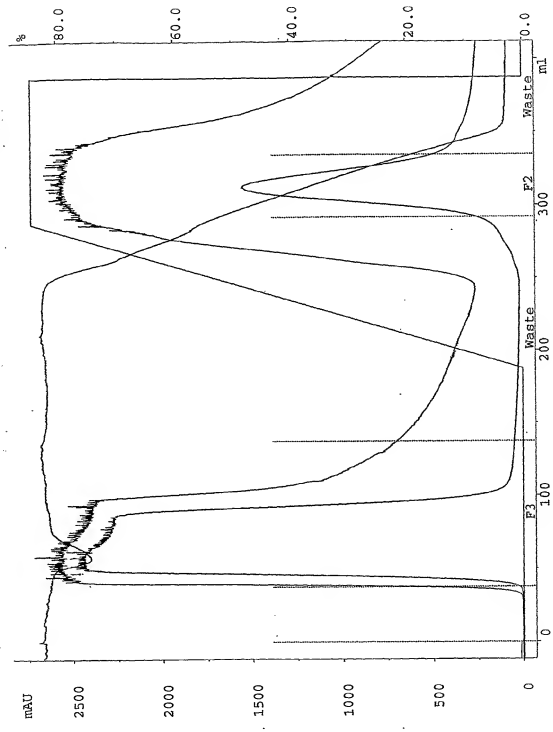
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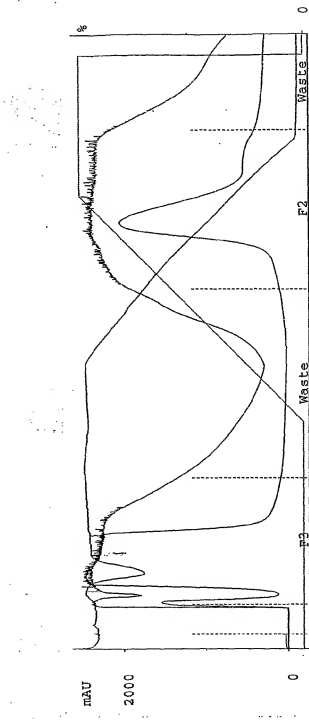
Figure 15



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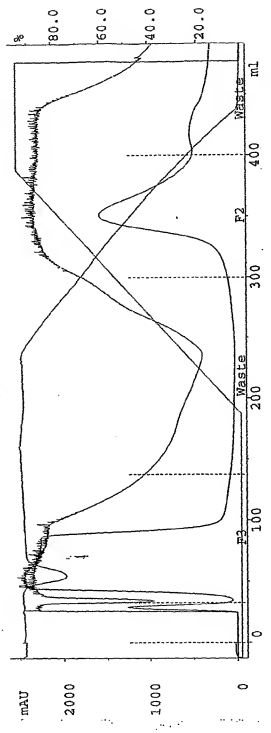
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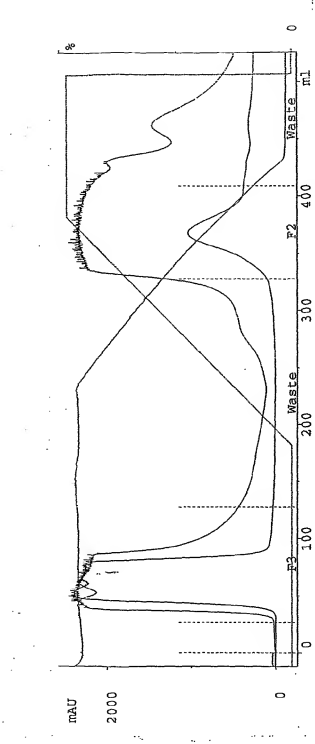

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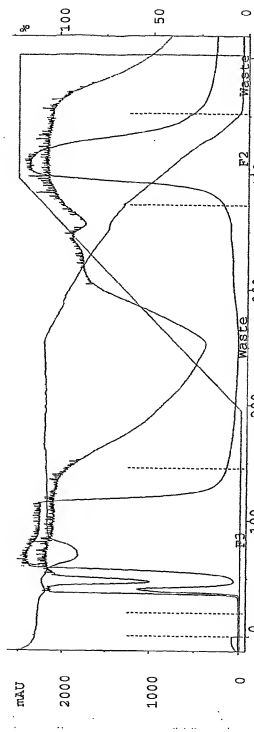

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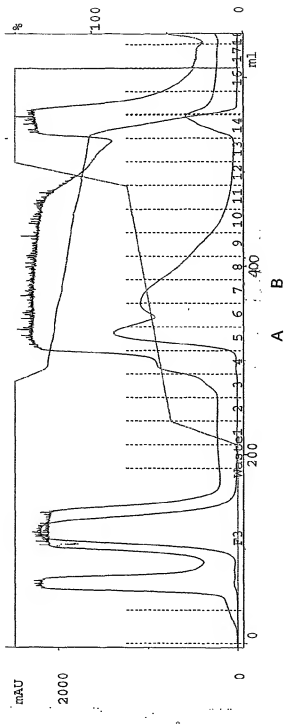

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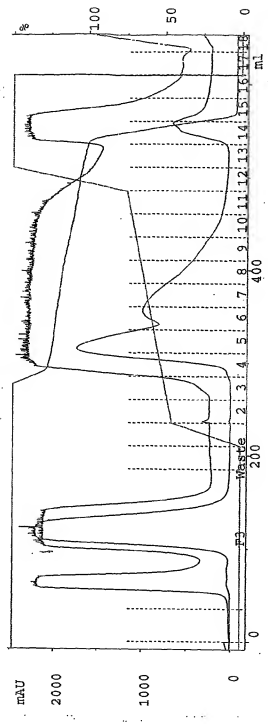
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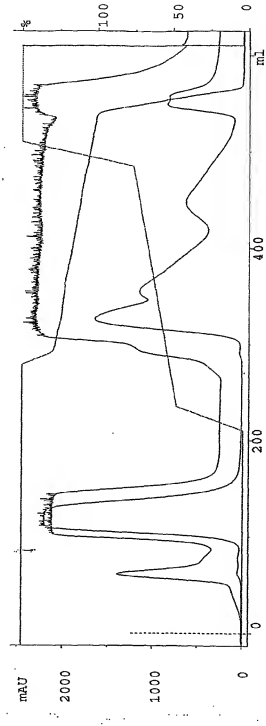

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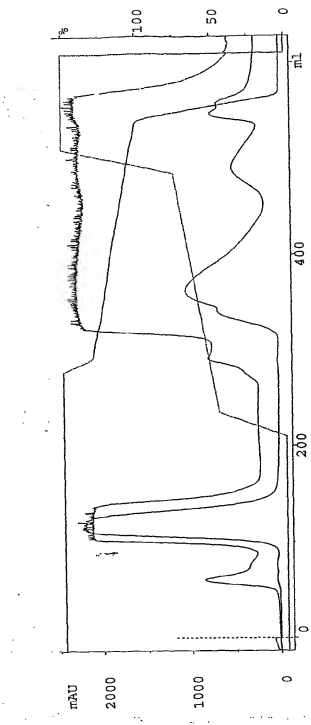

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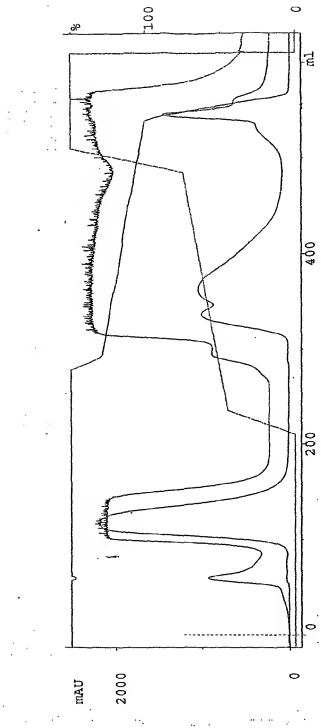

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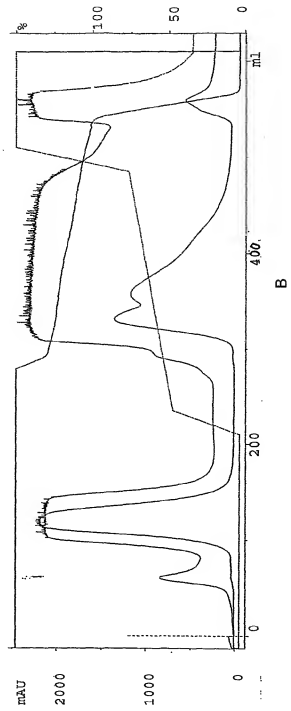

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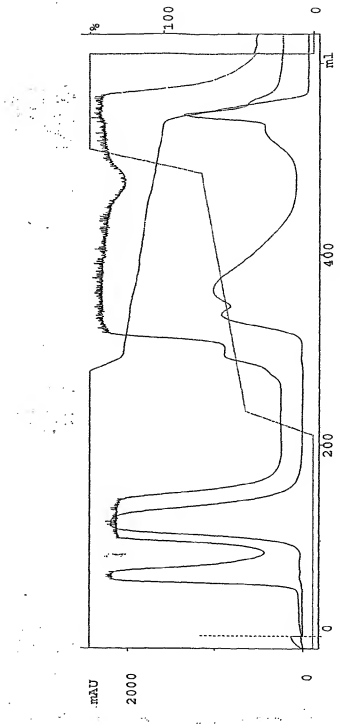

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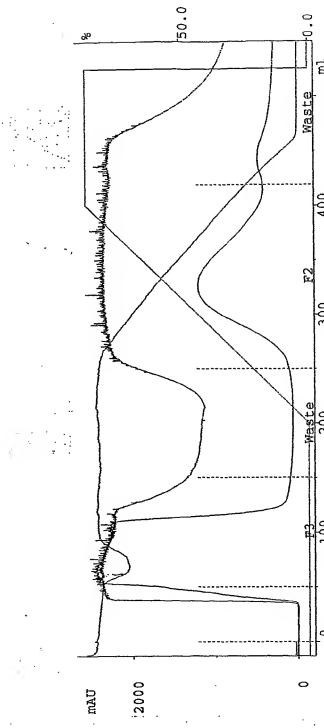

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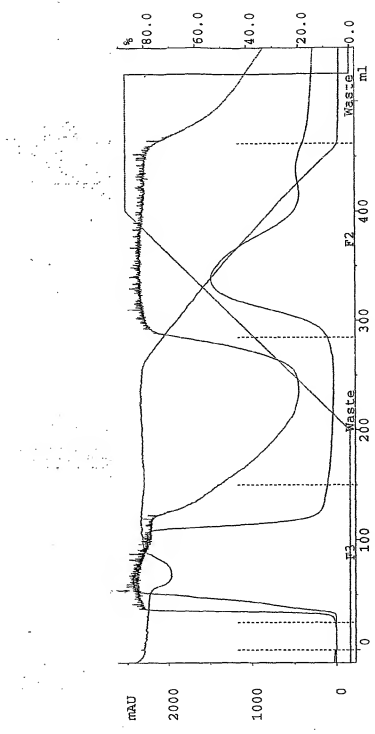
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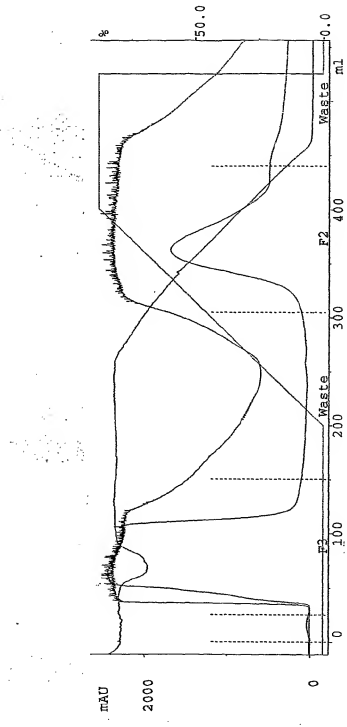

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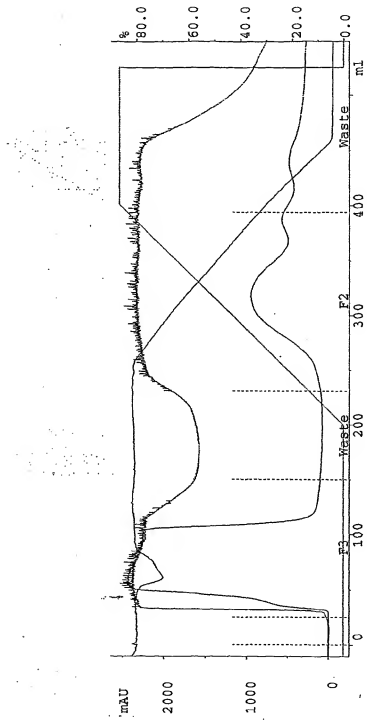
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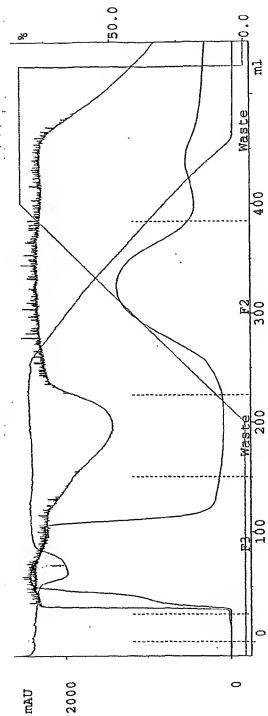
Figure 30



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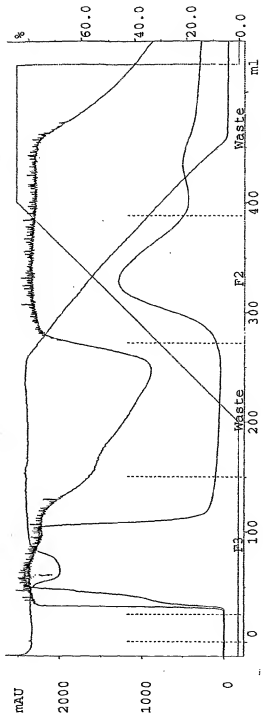
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Figure 32

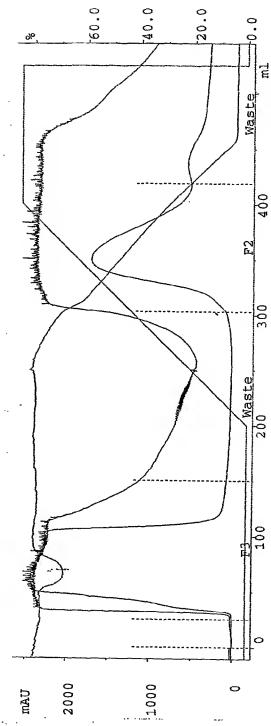
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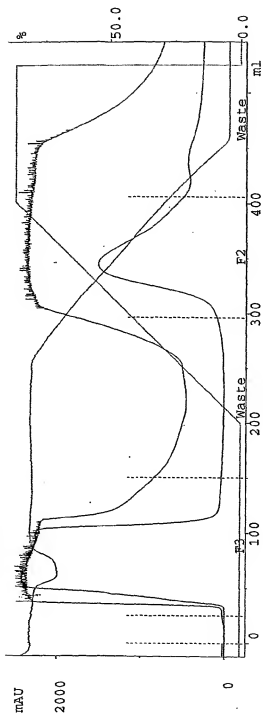
Figure 33



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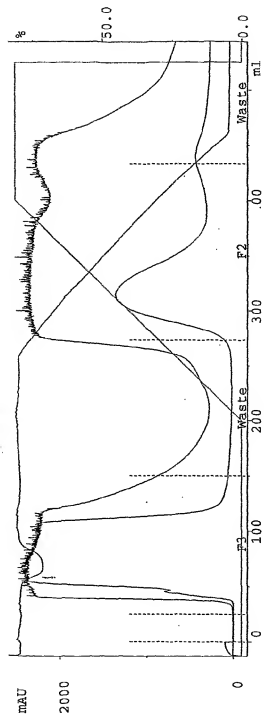

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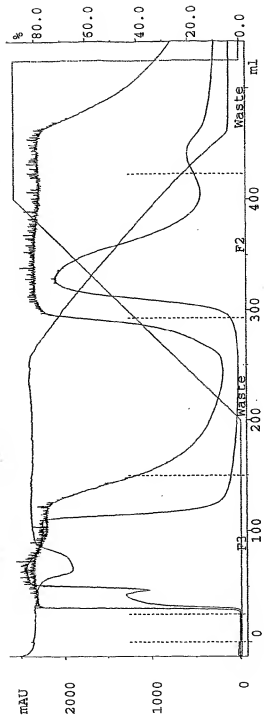
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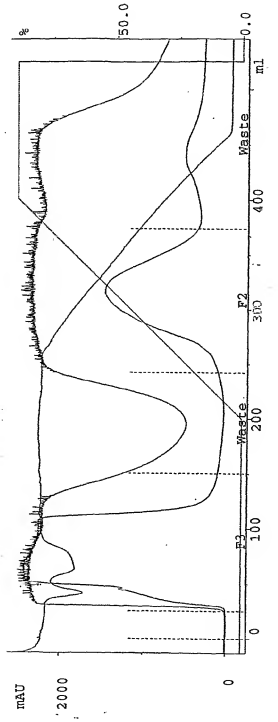
Figure 36



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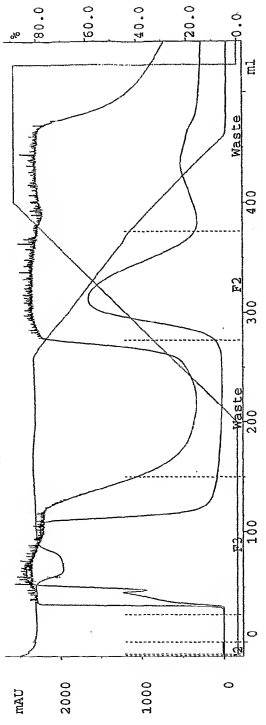
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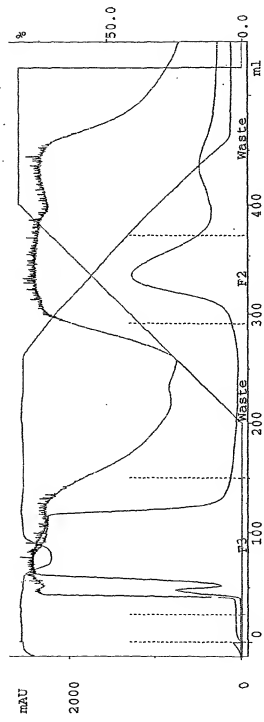
Waste

Figure 38



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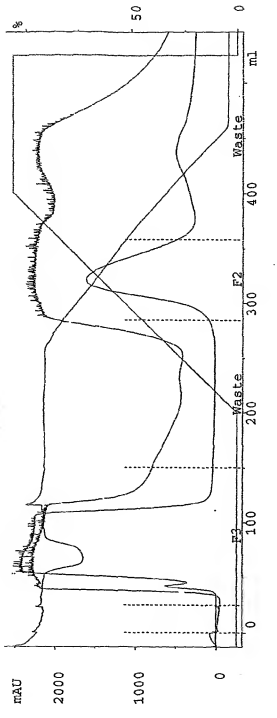

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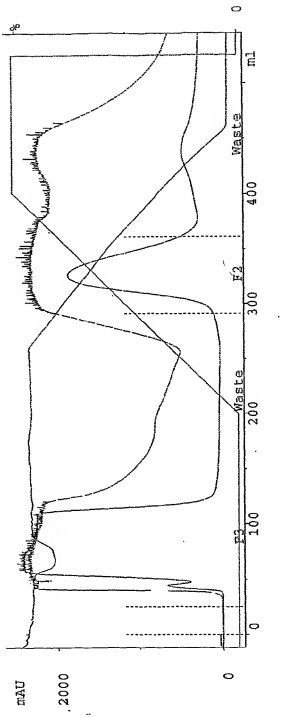
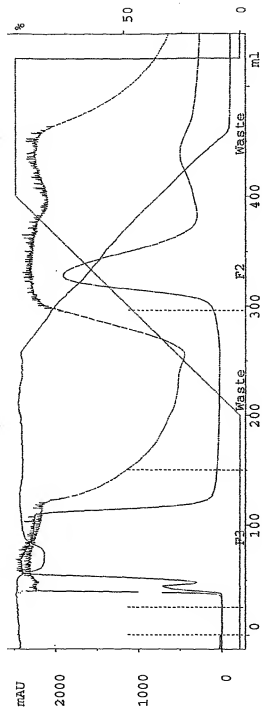


Figure 41



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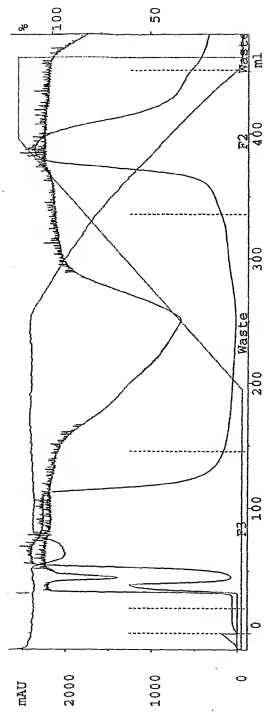

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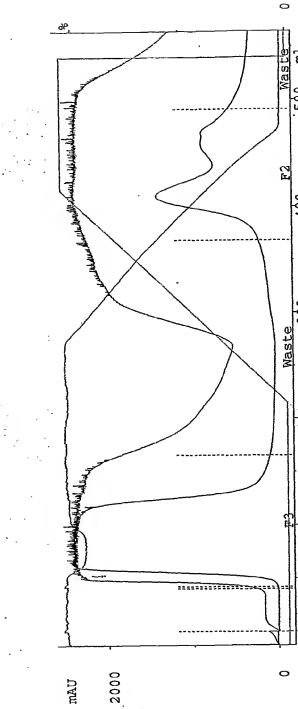

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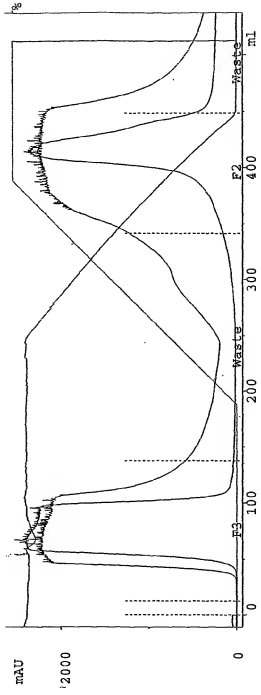
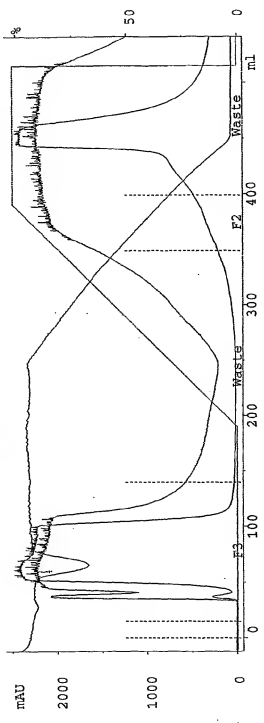


Figure 45



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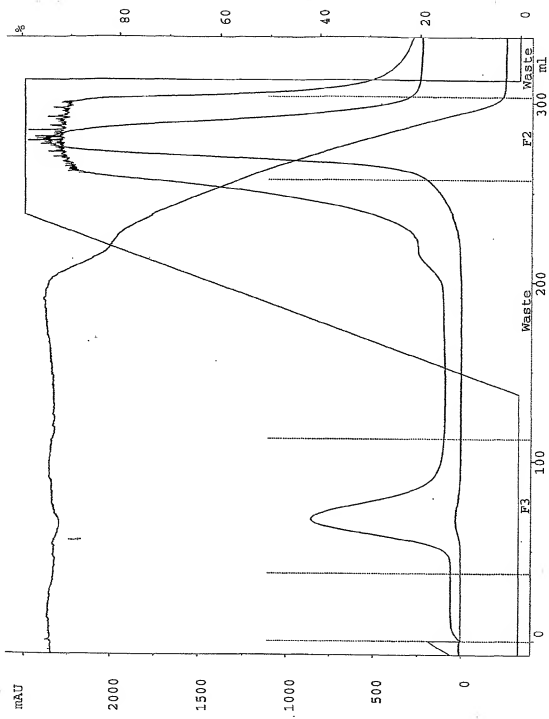
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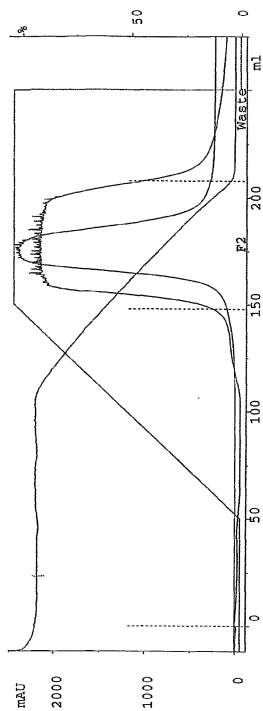
Figure 47



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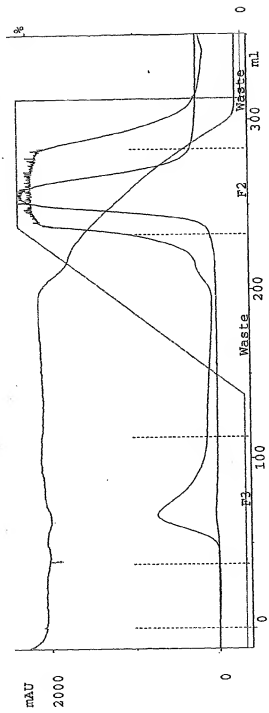

Figure 49

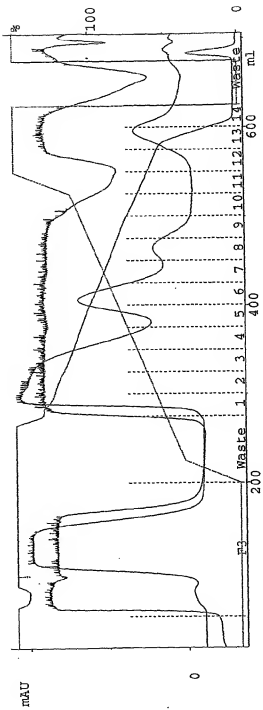

Figure 50

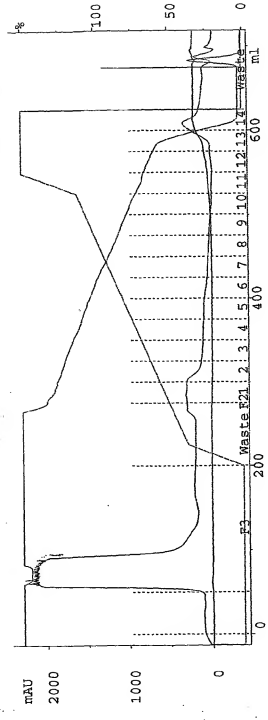
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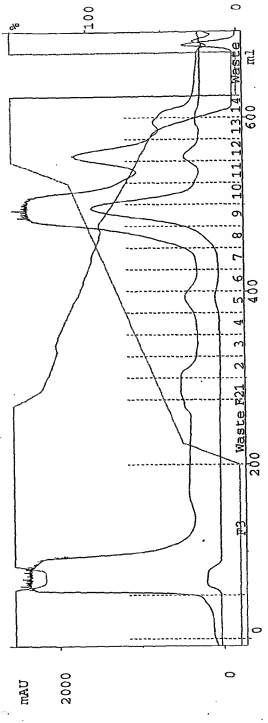
Figure 51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mAU
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Figure 52

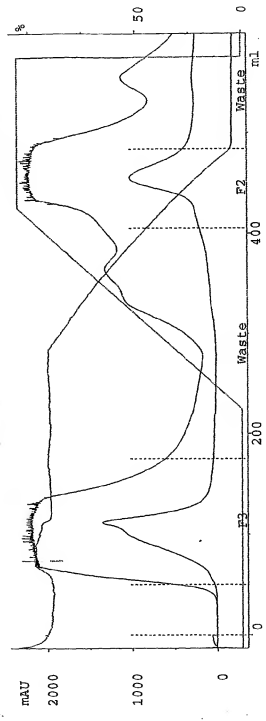
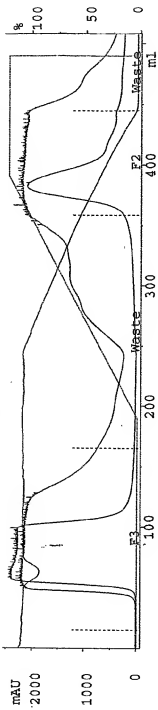


Figure 53



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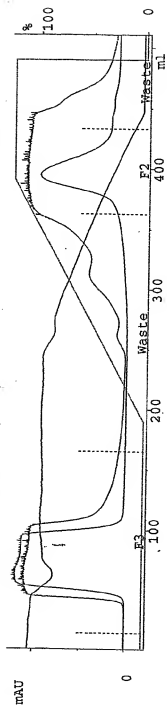

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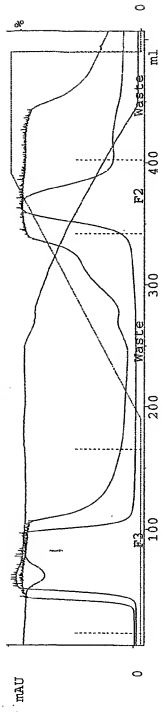

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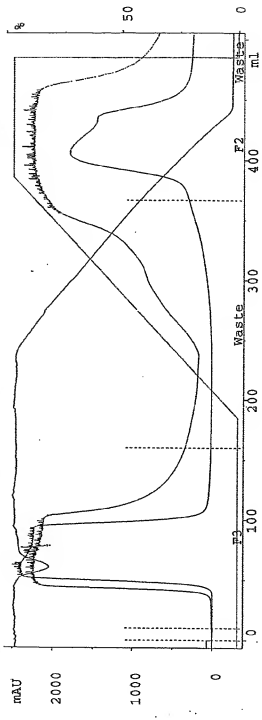

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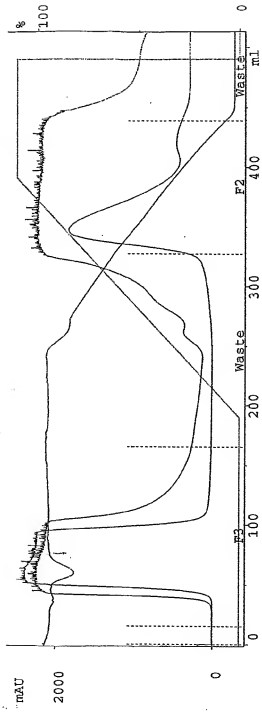

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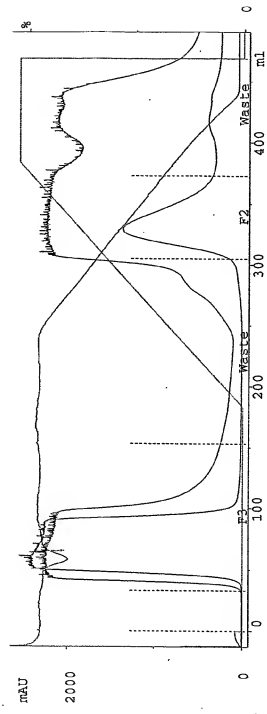

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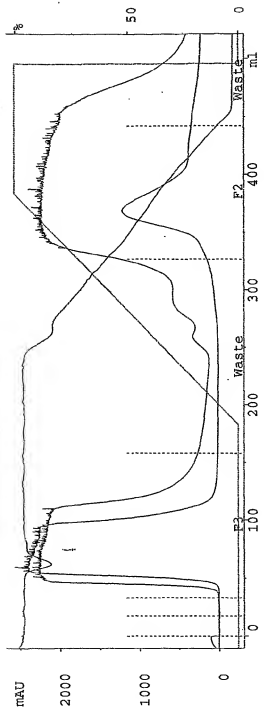

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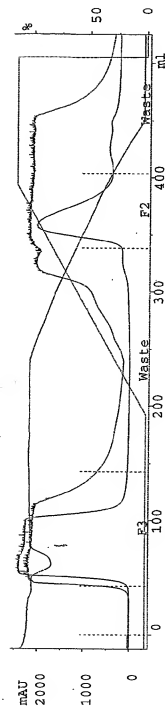

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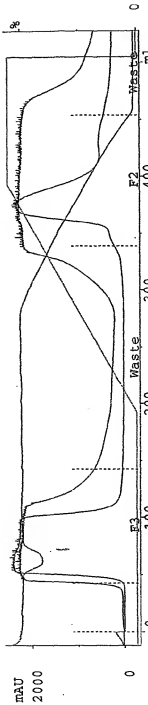
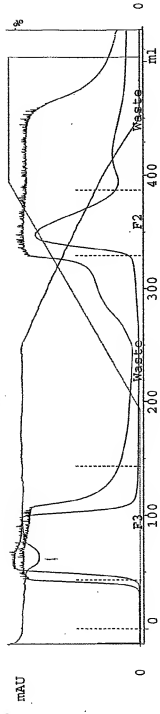


Figure 62



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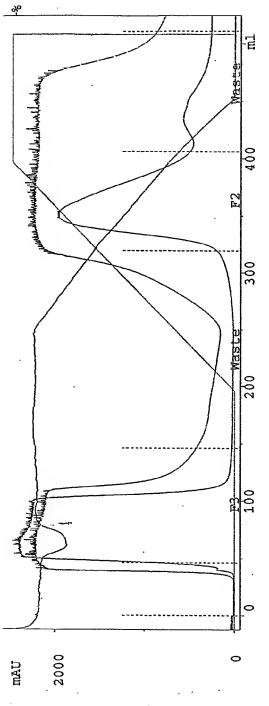

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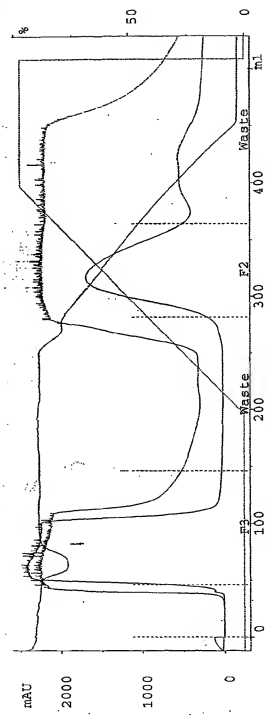

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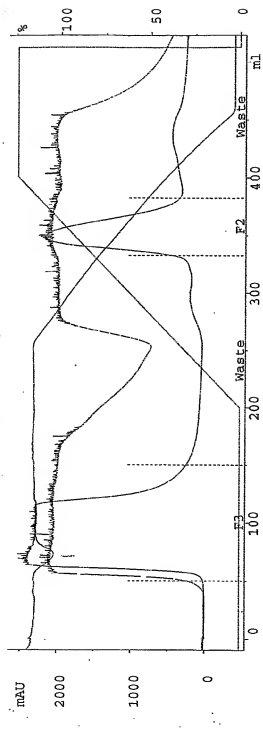

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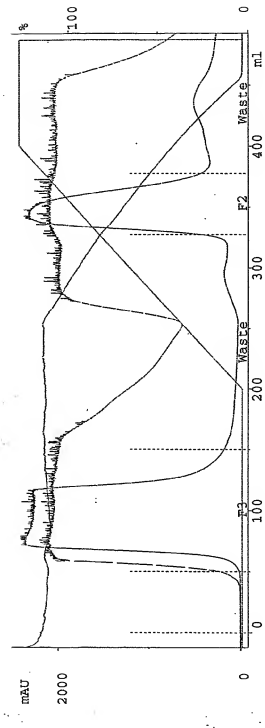

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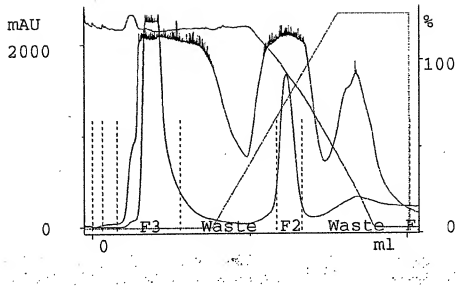

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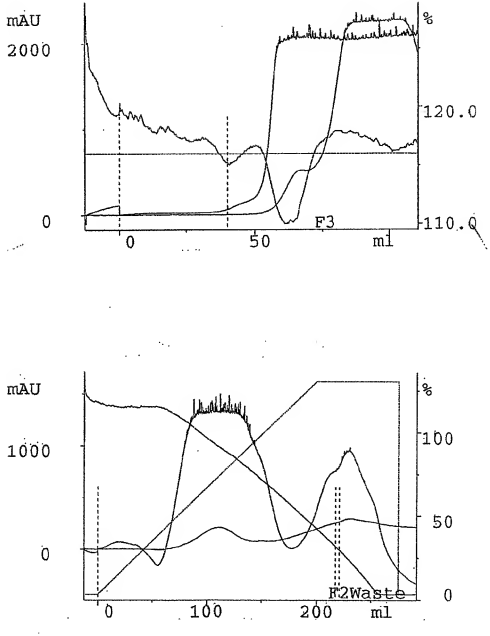
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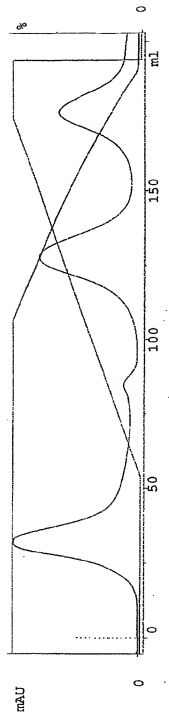
Figure 69



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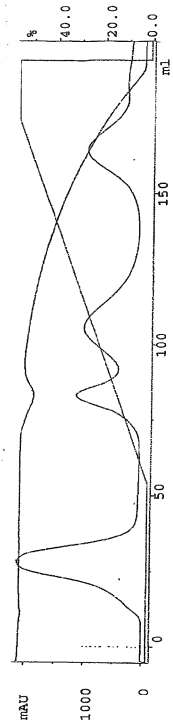

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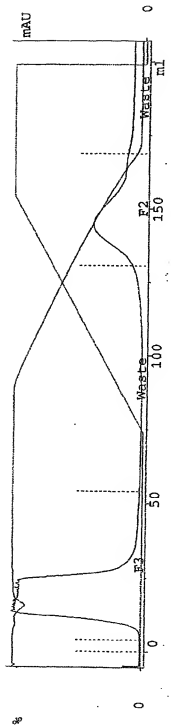

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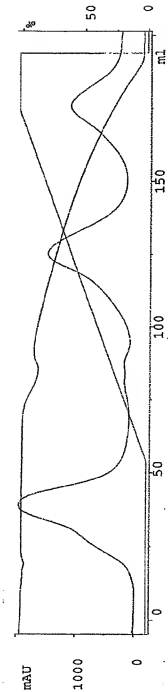

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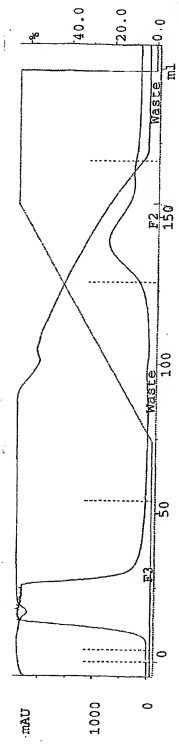

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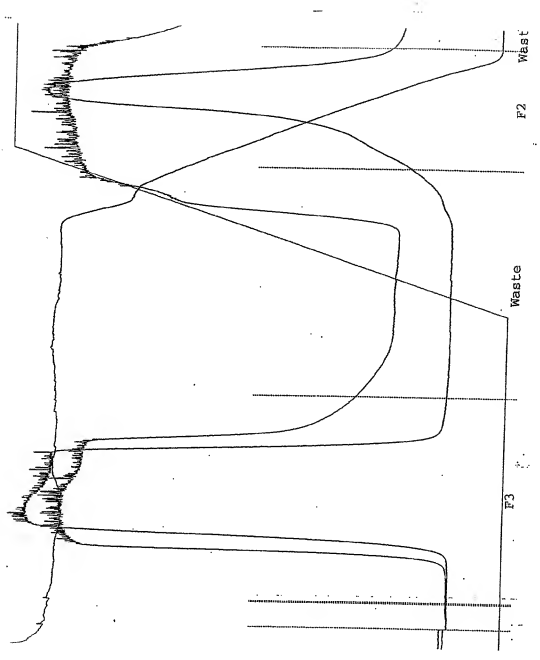

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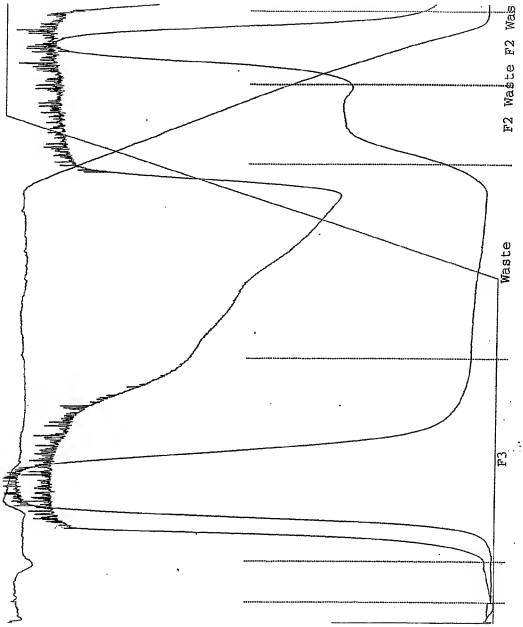

Figure 76

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2005/000614

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7): C07K 19/00, B01D 15/08, C07K 14/76, C07K1/ 20, A61K 47/48, A61K 47/42

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(7): C07K 19/00, B01D 15/08, C07K 14/76, C07K1/ 20, A61K 47/48, A61K 47/42

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used)
PubMed, Canadian Patent Database, Delphion, Keywords: HSA, albumin, conjugate, HIC, hydrophobic interaction chromatography, butyl-SEPHAROSE, phenyl-SEPHAROSE, exendin, GLP-1, insulin, ANP, sulfate, hydrophobic support, purification

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MARIANI M et al. "A Competitive Enzyme-Linked Immunosorbent Assay for Measuring the Levels of Serum Antibody to *Haemophilus influensae* Type B", CLINICAL AND DIAGNOSTIC LABORATORY IMMUNOLOGY, September 1998, vol. 5, no. 5, pages 667-674 materials and methods on pages 667-668	1, 6-8, 16-18 and 20-22

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 July 2005 (22-07-2005)	01 September 2005 (01.09.2005)
Name and mailing address of the ISA/CA Canadian Intellectual Property Office Place du Portage I, C114 - 1st Floor, Box PCT 50 Victoria Street Gatineau, Quebec K1A 0C9 Facsimile No.: 001(819)953-2476	Authorized officer Nicole Harris (819) 997-4541

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	LEGER R et al. "Identification of CJC-1131-Albumin Bioconjugate as a Stable and Bioactive GLP-1(7-36) Analog", BIOORGANIC AND MEDICINAL CHEMISTRY LETTERS 6 September 2004, vol. 14, no. 17, pages 4395-4398 whole document	1-8, 11-13, 16-22
P, A	THIBAUDEAU K et al. "Synthesis and Evaluation of Insulin-Human Serum Albumin Conjugates", BIOCONJUGATE CHEMISTRY July-August 2005, vol. 16, no. 4, pages 1000-1008	
A	DIAS-CABRAL AC et al. "Effect of Salts and Temperature on the Absorption of Bovine Serum Albumin on Polypropylene Glycol-Sepharose Under Linear and Overloaded Chromatographic Conditions", JOURNAL OF CHROMATOGRAPHY A 14 November 2003, vol. 1018, pages 137-153	
A	HOLMES DL et al. "Site Specific 1:1 Opioid:Albumin Conjugate with *in vitro* Activity and Long *in vivo* Duration" BIOCONJUGATE CHEMISTRY July-August 2000, vol. 11, no. 4, pages 439-444	
P, A	JETTE L et al. "Human Growth Hormone-Releasing Factor (hGRF)1-19 Albumin Bioconjugates Activate the GRF Receptor on the Anterior Pituitary in Rats: Identification of CJC-1295 as a Long - Lasting GRF Analog", ENDOCRINOLOGY July 2005, published on-line 7 April 2005, vol. 146, no. 7, pages 3052-3058	
A	LEGER et al. "Synthesis and *in vitro* Analysis of Atrial Nautriuretic Peptide-Albumin Conjugates", BIOORGANIC AND MEDICINAL CHEMISTRY LETTERS, 20 October 2003, vol. 13, no. 20, pages 3571-3575	